HORMONAL REGULATION
OF
ENERGY METABOLISM

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Compiled and Edited by
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FOREWORD

In a day of bigness, the need emerges for round table, small group discussion of certain problems.

The Conference on Hormonal Regulation of Energy Metabolism was arranged by the Program Committee on the premise that such a need existed in this field. The Proceedings are presented in this volume.

The remarks of Dr. Joslin, the Dean of diabetologists, for several reasons are removed from their strict chronological sequence, and, like Abou ben Adhem's name, lead all the rest. The central focus of the Conference was not, officially, diabetes. Yet the disturbed metabolism found in the disease, and the known and suspected physiologic effects of insulin, found their way into most of the discussions. Throughout the Conference, Dr. Joslin's wisdom served as a warm and strong cement to bring together the various disciplines represented.

The Chairman takes this opportunity to acknowledge the invaluable assistance of many members of the Conference in the organization and editing of the Proceedings, particularly Drs. Friskey and Gordon.

THE ROAD AHEAD

By ELLIOTT P. JOSLIN

ALL OF US TODAY, concerned with the treatment of diabetes look forward with hope in our hearts. Think how much hope one gathers from a meeting such as this.

This book which I hold in my hand was the first treatise on the treatment of diabetes.* Rollo wrote it only a few years ago—1796, actually but four years before my grandfather was born. When you read it and compare the training to which he was exposed with that which you before me have had, one cannot help gathering hope. Dr. Root always emphasizes one of Josiah Royce's dicta, namely, "the fecundity of the aggregation." What an aggregation is assembled in this room!

Just a word about the status of diabetes as I see it today. It is quite real to me. I was born in the little New England town of Oxford, Massachusetts. On its Main Street there were three large and commodious houses, and of the six heads of families in those houses, five came down with diabetes. The one who did not was not obese. That made quite an impression upon me. Also, another impression was created, because across the street from our home lived Mr. George Fred Daniels, who never went to college and yet was an educated man with a scientific spirit. He wrote one of the best histories of New England towns. Oxford was settled just after the revocation of the Edict of Nantes in 1687. Six years later occurred a massacre by the Indians of the Johnson family in the town. Mr. George F. Daniels, as a boy, walking back and forth to school, was taught by his father to lay down a stone on the site of the massacre. The stones grew to a cairn and later were replaced by a monument. From that example, I began laying down stone after stone or, rather, recording one by one my diabetics who died.

* Rollo, John Cases of the Diabetes Mellitus, with the Results of the Trials of Certain Acids, and other Substances, in *Cure of the Lues Venerea*, 2nd Ed London, T Gillet for C. Dilly, 1798.

The data have been compiled by the Metropolitan Life Insurance Company. During the period from 1897 to 1914, the average patient I treated lived 4.9 years. Those with onset under 10 years lived 1.2 years. Today, the average patient lives 16 years and those with onset in the first decade of life who have died have averaged 20 years. Those with onset between 40 and 59 years live 16 years, and those with onset from 60 onwards, instead of living 4 years as formerly, now live 10. Meantime a great change in the age at death of diabetics has taken place. Thus, up to 1914, those in our series who died were on the average 44 years old, but now the average is 65 years.

Years ago one of my patients gave me a thousand dollars and with it, through the kindness of Miss Amelia Peabody, a life expectancy medal was created which we give to those patients who live longer with their diabetes than they were expected to live without it. As a matter of fact, Case 8 of my series, who lived in one of those Oxford houses, completed her full span of life with the Naunyn treatment. Through the years we have given many of these medals, possibly 2000, and only yesterday I was impressed by the gratitude shown by a patient who received one, and how he was using it to encourage another diabetic to take heart for a long life. The distribution of these medals became so common that later the Quarter Century Victory Medal was created. There are now 63 who, after 25 years, have passed the tests of ophthalmologists, roentgenologists and internists by being free from degenerative stigmata in the eyes, the blood vessels and the kidneys.

If one looks for the greatest common divisor in these patients who have done unusually well, it will be found to be the meticulous care which these patients have received. The first case had almost everything at his disposal—wonderful parents, one of our best nurses, who remained with him 7 years, funds sufficient to supply his needs and even allow him and his nurse alone to fly in his airplane from New Jersey to Cape Cod; and finally his marriage to a girl who spent months learning how to take care of diabetics. He had diet, exercise and insulin, plus what is needed today, namely, opportunity and a zeal to follow treatment. His life has stimulated us to build the Hospital Teaching Clinic, where teaching will take precedence over nursing. In this way,

money can be saved, and at reduced costs we can invite back our patients so that they, too, will have an opportunity to learn how to be medal cases. We hope that the results we obtain in the Hospital Teaching Clinic will be so good that it will lead to similar opportunities being created for diabetic patients in every hospital in the world. Until now the young diabetic has carried on his back the expense for the care of the old and seriously complicated diabetic, whose future holds little chance for rehabilitation. Today there exists no discrimination in hospitals between the cost for the care of the young and ambulatory diabetic with years of possible activity ahead, requiring chiefly teaching, and the old, pathetic patient whose life is nearly at an end, for whom almost hourly nursing is necessary.

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I

CERTAIN ASPECTS OF HORMONAL REGULATION OF CARBOHYDRATE METABOLISM

By DeWitt Stetten, Jr.

I do not plan to present original or new contribution, but rather to consider certain problems, certain areas of ignorance, possibly to provoke a few arguments.

We may start out with the idea that certain endocrines operate in the organism by controlling the rates of specific enzyme catalysts. This may not be universally true, but I believe it is foremost in the thoughts of many workers in the field. Right away we are up against an overwhelming difficulty. As was pointed out by some of the speakers at a recent meeting, there is not a single enzyme-catalyzed reaction for which we can today write a mechanism that is satisfactory to the organic chemist. In other words, we are talking about the regulation of reactions that we don't understand. One of the obvious reasons we cannot write mechanisms for enzyme-catalyzed reactions is that, thus far, we do not know the structure of the enzyme. Before the organic chemist can write a mechanism for a reaction, he must obviously know in some detail, the structures of the reactants. It follows almost necessarily, that since we don't know the mechanism of any enzyme-catalyzed reactions, we do not know the mechanism of the endocrine control of such a reaction. As a matter of fact I believe there is no endocrine-regulated phenomenon about which one would find even a reasonable degree of concordance as to how it operates.

In contrast to the situation of the enzymologists, the endocrinologist has made considerable progress in elucidating the structures of the compounds in which he is predominantly interested. The structures of epinephrine, thyroxin and insulin can today be written, but even with these compounds there are large areas of ignorance. The structures that have been established are those of compounds which have been extracted from the endocrine

Levine's laboratory; also in Parks and other laboratories, suggesting that the action of insulin is related to the transport of glucose, chemically unaltered or minimally altered, from the extra-cellular to the intra-cellular compartment. I believe this latter to be the best working hypothesis of insulin action that we have today. I would point out however that it fails to explain certain data in the literature. As an example I might mention a report from Burk's laboratory, dealing with a response to insulin in the homogenate of a mouse sarcoma, in which no cells and no cell membranes were present. This insulin response is similar, though perhaps not identical, to the effect of insulin that might be anticipated in an intact tissue. It is therefore possible that the hypothesis from the beautifully executed experiments of Dr. Levine and his colleagues may not explain *all* of the manifestations of insulin action. In any event, I believe that we can focus on the entry of glucose, and its initial phosphorylation, as the *areas* in which insulin acts. I would repeat that the *mechanism* of insulin action is today, as far as I am aware, completely unknown; this despite the fact that in this particular case we do know the structure of the hormone in question.

The *phosphorylase* reaction appears to be under rather elaborate endocrine control. At least two materials, *epinephrine* and *glucagon* have been implicated by Sutherland as affecting the activity of phosphorylase in certain tissues Epinephrine seems to favor the reactivation of inactive phosphorylase in liver and in muscle. Glucagon apparently does something similar, but most workers feel that its activity is entirely or almost entirely restricted to the liver. It seems odd that the body should go to the trouble of making two hormones, one of which acts in two places, the other acting in the same fashion only in one place. It is worth noting that whereas the normal venous drainage of the adrenal is into the systemic blood, the normal venous drainage of the pancreas is via the portal vein into the liver. Bornstein has stated that glucagon is quantitatively removed by one passage through the perfused liver. Apparently glucagon not only does not affect phosphorylase in muscle, it probably never gets there. The differences in phosphorylase in liver and muscle are also of interest to us

Another reaction which has recently attracted attention is the

glands after more or less vigorous treatment. There is I believe, no assurance in any single case that the product whose structure is written in the text book is indeed that of the native product that occurs in the gland from which it is extracted. There is also, in most cases, no assurance that the product which circulates in the blood is identical chemically with the product which is isolated from the gland. In this area the thyroid physiologists have perhaps progressed further than the insulin physiologists.

Insulin, generated in the pancreas, must, in all probability, pass through the liver before it gets to its most important target organ, the skeletal muscle. There is no clear evidence that the material that goes into the liver is the same as that which comes out of the liver. As a matter of fact, much of the recent speculation and experimentation would suggest that insulin is altered in the course of its passage through the liver. Unfortunately most of the therapeutic and experimental applications of insulin have involved its administration not into the portal blood, but into peripheral blood It is therefore difficult to ascertain that the insulin we are injecting is arriving in the same condition as is the insulin which arrives at the muscle after intrinsic production in the pancreas.

Then there is another problem, namely, whatever the form in which the hormone travels in the blood, what is the form in which it operates on the target organ? Here again thyroid physiologists, I think, have led the way. I have no doubt that we will hear something about this from Dr Gross before the meeting is over. I believe that there is no assurance that the material which produces the actual effect on the target organ, is chemically identical with the material which travels to the target organ through the vascular tree.

Whereas we cannot say much about intimate mechanisms of most hormonal effects, there has been considerable speculation and experimentation designed to determine the sites of action of several of the endocrine agents.

The site of action, or at least a major site of action of insulin is generally believed today to be early in the course of utilization of glucose, and possibly other sugars. Attention was focused upon this by the work in the St. Louis laboratories. Over the past few years there has been an impressive accumulation of evidence from

instance, it is held that the concentration of the ions which are represented in the bone mineral in the body fluid is at least in part a reflection of equilibration, in the physical chemical sense, of a solution in contact with a solid phase, the solution being essentially saturated with the constituent of the solid phase. However, in most of the situations which one explores biochemically, he finds that in the living organism operations are running quite remote from equilibrium. Thus the phrase that one hears so frequently: "The amino acids of the body are in equilibrium with the proteins of the body" is anathema to some of us. This is quite obviously not true. Most of the nitrogen of the body is in the form of polypeptide or protein. If polypeptide is exposed to water it is hydrolized spontaneously. If one takes a mixture of amino acids in water, no matter how long one waits he does not get an appreciable yield of polypeptides. The equilibrium here is entirely in favor of hydrolytic products.

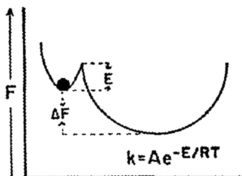


FIG. 2 Kinetic stability.

Therefore, the relationship of the amino acids of the body and the proteins of the body is certainly not a reflection of equilibrium forces.

The second situation that one considers when one wishes to explain constancy of composition is something which has been called "Kinetic stability." The ball in this case has been trapped in a crevasse and cannot readily roll to its position of maximum stability at the bottom of the valley (Fig. 2) It must overcome a certain energy "hump" which we call the energy of activation, before it can roll down. This model breaks down when we consider the situation in chemistry, because, instead of dealing with a single ball, we are dealing with a statistical population of molecules whose energies are not uniform but are distributed However, in this population there will always be a fraction of molecules having energy in excess of E and the magnitude of that fraction will determine the rate in which the balls can escape from

glucose-6-phosphatase reaction. This enzyme has been shown by several workers to increase in activity, and presumably in abundance, in the diabetic state A difficulty in which the diabetic necessarily finds himself, stems from the fact that the reaction from glucose to glucose-6-phosphate is resricted, while the reaction from glucose-6-phosphate to glucose is exaggerated. It is quite obvious that the diabetic is hard put to generate everything which derives from glucose-6-phosphate.

So much for some of the more obvious relationships. The relationship of some other endocrines appear to be more subtle.

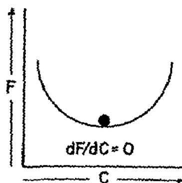


FIG. 1. Thermodynamic equilibrium

In trying to give "background" to (I hope) future discussion, I would like for a moment to consider one of the more baffling problems in biochemistry namely, why does the composition of the normal adult organism tend to remain constant? This is a problem which has intrigued physiologists since Claude Bernard's day. One frequently hears the word equilibrium used to explain such constancy. "Equilibrium" is a word which is completely respectable, and is well and precisely defined by the physical chemist. It is the condition of minimal free energy. It is the condition from which there is no escape unless work is done on a system It is often represented by a diagram of a ball resting in the bottom of a valley. The ball cannot possibly escape unless outside work is caused to drive the ball out of the point of maximum stability (Fig 1). Equilibrium is the first thought that the chemist will have when he encounters a system of invarient composition. He will say, "Let us see whether this system is in equilibrium." If it is in equilibrium, this will explain why its composition is invariant, and there are certain tests by which the existence of equilibrium may be established I shall not trouble you with these tests. I will only point out that in the body of the mammal there are, probably, certain situations in which the constancy of composition is contributed to by equilibrium considerations. For

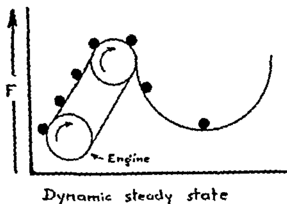


FIG. 3.

of the body are continuously breaking down, and enzymes for their breakdown are continuously available. In order to maintain a constant supply, one must picture a nice balance between the rate of regeneration of such molecules, and the rate of spontaneous or enzyme-catalyzed decay. We must postulate an engine (Fig. 3) (and I was happy when our chairman used the word "engine" earlier this morning). We can today give some kind of picture of what this engine looks like. The engine derives its power from the potential difference between inorganic phosphate, Pi, and the high phosphate compounds represented as ~ P (Fig. 4). There is a

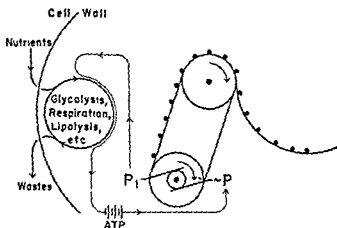


FIG. 4. The relationships of catabolic processes, coupled phosphorylations and the dynamic steady state.

the crevasse and roll to the bottom of the valley. That fraction is represented by the exponential term $e^{-E/RT}$ where E is the energy of activation, and T is the absolute temperature. If E is large and T is small, that is if the temperature is low, the frequency with which molecules escape and achieve their minimum energy level will be low. If the temperature is elevated or if the energy of activation is diminished this frequency will increase. It is because the energy of activation of hydrolysis of peptide bonds is high, that insulin solutions, while obviously not in equilibrium, are still relatively stable, particularly at low temperatures. Does this sort of thing happen in the body? The answer is yes, of course it does. But it happens less in the body than in the bottle on the shelf of the laboratory because it may be said that enzymes in general have the function of diminishing the value of E. Enzymes in general reduce the energies of activations of the reactions which they catalyze. This is the way in which catalysis is effected. Consequently, whereas a bottle of glucose on the shelf, although not in equilibrium with atmospheric oxygen remains chemically constant, this same glucose when dissolved in yeast juice or when mixed with a muscle mince disappears rapidly with evolution of products of degradation, depending upon the enzymes represented. Consequently in a medium rich in such catalysts, kinetic stability is less than in a medium poor in such catalysts. Still there are situations where we find amazing kinetic stability. One such situation for instance is in the collagen of the extra-cellular component of connective tissue. Here we have a protein which is highly insoluble, which is in a situation apparently fairly remote from proteolytic enzymes capable of attacking it, the intracellular cathepsins apparently having little access to collagens. There have been experiments, particularly by Neuburger, indicating that the life expectancy of collagen molecules, in contrast to that of the serum albumin molecule, is very long; even greater than the life expectancy of the animal which harbors it. In other words a molecule of collagen once laid down has an excellent chance of outlasting its host. We can attribute the constancy of this particular protein to kinetic stability.

However, most of the body constituents of greatest interest are in a state of continuous turnover. Particularly the larger molecules

stimulation; and (T) and (HT), the concentration of unoccupied and occupied sites on the target organ. By a transformation similar to that of Lineweaver and Burk, an expression is reached: $(H)/(HT) = K/Q - (H)/Q$ wherein $Q = (T) + (HT)$, and is a measure of the total concentration of target organ sites where hormone may reside. If the response to hormone in solution is, under given circumstances, proportional to the concentration of HT (target sites occupied by hormone), a straight line should result when hormone concentration divided by response $(H)/(HT)$, is plotted against hormone concentration (H). The slope of such a line will equal $1/Q$ and the intercept $(H = O)$, should equal K/Q.

Entirely satisfactory data for testing of this hypothesis have not been found in the literature. Application of these equations to certain published data have, however, been considered encouraging. In a qualitative sense, the foregoing argument accounts for the fact that whereas, at low concentration, the responses to many hormones are proportional to concentration, maximal responses have been reported to many hormones. Increases in hormone concentration beyond those required to elicit maximal response are without further effect.

Analysis of hormone action in these terms will have to await accumulation of suitable data in which a pure response to a hormone is measured in a relatively uncomplicated system *in vitro*, over a wide range of known hormone concentrations. Should it prove valid, such analysis may permit comparison of various members of a family of related hormones, as well as classification of hormone antagonists as "competitive" or "non-competitive." It is offered here as a possible direction of development in the area of experimental endocrinology.

DISCUSSION

DR STADIE: You have done a job, Dr. Stetten, which I wish I had done, namely, to formulate the phenomenon of "insulin binding" in mathematical terms. For the benefit of those of you who are not familiar with the subject, I shall review briefly some of the work on this subject which Haugaard and I published some years ago. When an isolated surviving rat diaphragm was equilibrated

storage battery of ~ P represented by adenosine triphosphate. As the engine runs, this latter discharges. The processes of glycolysis, respiration, lypolysis etc. all consume capital Pi and regenerate ~ P. In so far as this is accomplished, the battery is recharged. However, the *coupling* of oxidate processes to the phosphorylative processes is a loose one. *Uncoupling* is affected by a variety of agents. A role which has been attributed to thyroxin is that of accelerating uncoupling of oxidative phosphorylation.

I should now like to disgress for a few moments to consider certain quantitative aspects of hormonal activity. The low concentrations of physiologically active hormones in the circulating blood might suggest that the cells of the target organ possess a selective mechanism of concentration of hormone. Some evidence indicating the nature of such a mechanism is at hand from the work of Stadie and his collaborators, who have shown that not only muscle, but also adipose and mammary tissues possess the capacity of adsorbing and firmly binding insulin from solution. The situation is not dissimilar to that described by the Michaelis-Menten concept, that the rate of an enzyme catalyzed reaction is determined by the instantaneous concentration of an enzyme-substrate complex. Translating this concept into endocrinological terms it may be postulated that the effect of a hormone is determined by the instantaneous quantity of hormone bound by the effector or target organ. It is of interest to speculate from the assumption that the response in the target organ is in effect determined by the abundance of target organ site occupied by hormone, (HT). Employing a derivation analogous to that used by Michaelis and Menten to describe enzyme kinetics, or by Langmuir to describe the phenomenon of adsorption, an expression may be derived defining the equilibrium of the reaction:

$$\mathrm{H} \rightleftharpoons \mathrm{H} + \mathrm{T}$$

$$\mathrm{K} = \frac{(\mathrm{H})(\mathrm{T})}{(\mathrm{HT})} = (\mathrm{H})^{1/2}$$

where (H) is the molar concentration of hormone in solution; $(\mathrm{H})^{1/2}$ is that concentration required to produce half of maximal

brated in a solution containing insulin. The concentration of insulin and the fixation period are varied in order to vary the amount of insulin which will be bound to the diaphragm. The hemidiaphragms are then washed and the amount of bound insulin is estimated in two ways: (1) by its biological activity in causing extra glycogen synthesis (compared to controls), during a 90 minute period of aerobic equilibration with glucose. Obviously such "insulin effects" give values which, although a function of the bound insulin, cannot measure its absolute amount, and (2) by analysis of the diaphragm for radioactivity, which yields a precise quantitative value for the concentration of bound insulin.

The first problem we studied with labeled insulin was whether, as we had predicted, insulin combines firmly with diaphragm during a short exposure, and resists the dissociating action of washing.

In these experiments hemidiaphragms were immersed in medium containing insulin at different concentrations for 1 minute, washed briefly in three changes of medium, and then washed for prolonged periods of time. Finally they were analyzed for isotopic insulin. There was some dissociation in the beginning but further washing up to 60 minutes had no additional effect. This residually firmly bound insulin is a function of the concentration of insulin during the 1 minute period of exposure, and is biologically active.

The bound insulin is a function of the insulin concentration during the fixation period. There is an increase of the bound insulin until a saturation level is reached. Apparently it is possible to saturate the ability of the normal rat diaphragm to bind insulin at about 1.5 microgram per gram of tissue.

The use of isotopically labeled insulin has enabled us in the same experiment to measure bound insulin and the biological effect of insulin.

The *insulin effect* on glycogen synthesis of normal rat diaphragms, is plotted as a function of bound insulin. The statistically calculated regression line gives the effect of a unit mass of insulin bound to a gm. of diaphragm in accelerating above the normal rate the reactions which lead to the synthesis of glycogen. This may be expressed in several ways. In molecular terms it is 13,000 moles of glucose per mole (MW = 48,000) of insulin per

momentarily in a solution containing insulin (as little as 10 seconds sufficed), it subsequently responded by extra glycogen synthesis from glucose without further contact with the hormone. Since this effect persisted after prolonged washing of the diaphragm and, as shown by appropriate controls, could not be due to insulin in solution, it was concluded that during the brief exposure to insulin the hormone had been bound to cellular elements of the diaphragm by forces not yet understood, and that in this bound form it exerted its characteristic effect on metabolic reactions. It was later shown that this phenomenon could be demonstrated in other tissues, namely, lactating mammary gland, liver and adipose tissue. In each case a characteristic metabolic reaction was influenced by bound insulin. In addition it was shown that the metabolic effect of the bound insulin was influenced by the endocrine state of the experimental animal, when this was altered by removal of endocrine organs or injection of potent endocrine principles prior to the removal of the tissue for the experiment. The apparent generality of this phenomenon led us to entertain the hypothesis that the binding of insulin on loci of intact tissue was a prerequisite for its physiological activity.

In order to study this phenomenon in a more systematic fashion we subsequently prepared and used isotopically labeled insulin. This has enabled us to determine quantitatively the actual amount of insulin bound and to correlate this with its biological effect.

Two derivatives of insulin have been prepared: (1) insulin sulfate, with S^{35} in the sulfate group attached to aliphatic hydroxyl groups, and (2) insulin with radioactive iodine in the tyrosine residues. Specific activity ranged from 2 to 8 million cpm. per mg., enabling determination of bound insulin of the order of 0.01 microgram. The biological activity of these preparations is apparently unchanged, and the close quantitative agreement obtained using both preparations in identical experiments, gives assurance of the validity of the results.

The standard technique which Haugaard and I developed for experiments on the combination of insulin in rat diaphragm involved the following: (1) fixation, (2) washing, (3) assay, (4) analysis. In the first or fixation period one hemidiaphragm is equili-

pophysectomized rats. We may conclude from these data that removal of pituitary factors has permitted the full accelerating effect of insulin on the reactions concerned in the synthesis of glycogen to be manifest. This is equivalent to saying that "pituitary factors modify the *action* per se of insulin itself, rather than the *metabolic systems* concerned with glycogen synthesis"—a matter which has been the subject of controversy in the literature.

One more point comes to me: We tried to demonstrate the phenomenon of binding in other tissues. We were able to do so in the case of the mammary glands and adipose tissue itself. We were unsuccessful in the case of the kidney. We also did some experiments trying to demonstrate an effect upon normal liver slices in the ability to incorporate acetate into higher fatty acids but were unsuccessful.

DR. GORDON: I would like to ask Dr. Stadie if he tried gentle reduction of S-S groups to SH to see if that would affect the binding.

DR. STADIE: No we did no experiments of that sort.

DR. WICK: Was there any difference between fed and fasted animals on the binding of insulin?

DR. STADIE: We always fast our animals for 18 hours. We did not study the effect of longer fasting. We never did any systematic studies on fed animals.

DR. LEVINE: Dr. Stadie, in the hypophysectomized diaphragms you had a greater effect with the same amount of bound insulin?

DR. STADIE: That's right.

DR. LEVINE: The pituitary does not seem to interfere with the binding.

DR. STADIE: That was our opinion. We were never able to demonstrate any effect of growth hormone upon insulin binding by the rat diaphragm. However we got the impression that there was

strong inhibitor. Now the new experiments of Dr. Levine, Park and so on indicate that that actually is the site because the concentration of free glucose in the cells seems to be controlled, and once the glucose gets into the cells, the phosphorylation mechanism is assumed to be satisfactory.

DR. WICK: What does Dr. Stetten think about the insulin used in diaphragms? Is there any insulinase present or something else that would change the form of the insulin?

DR. STETTEN: I know of no evidence that there is insulinase in diaphragm. It has been demonstrated most frequently in the liver and kidneys, I believe. Drs. Schwartz and Mertz, in our Institute have one preliminary experiment in which the effect on blood glucose of intraportally injected insulin has been compared with that of insulin injected into a peripheral vein. The two glucose curves are superimposable. This very brief study has only been done twice. The insulin injected appears to get through the liver virtually unaltered with respect to its ultimate activity. If this is so, one is inclined to wonder what intrahepatic insulinase is doing, if anything, to the insulin which passes through the liver. It may be that this enzyme, although active against insulin in vitro, in the organism never has the opportunity to destroy insulin.

DR. RANDLE· I think there are a number of points in relation to kinetic interpretations of insulin binding which should be emphasized. Personally I think the action of insulin on isolated tissues such as the rat diaphragm is too complicated to be capable of kinetic interpretation—there are too many variables. One complicating factor is the ability of certain tissues such as muscle to inactivate insulin (Mirsky, I. A.: *Ciba Foundation Colloquia on Endocrinology*. Churchill, London. *6*, 263, 1953). It is possible too that tissues may bind insulin at more than one site, though the action of insulin on the metabolism of the tissue may occur at only one of the sites of binding. The evidence which Dr. Stadie has summarized suggests very strongly that insulin is bound by the site at which it acts. Chayen and Smith, and Ottaway have

a factor in crude APE extracts that actually did prevent binding. But we did not pursue this subject systematically.

DR. LEVINE: If in the hypophysectomized diaphragm, no more insulin is bound than under normal conditions, then the facilitation of glycogen deposition must be due to other causes. Would you care to speculate as to what those may be?

DR. STADIE: I think I should leave that to Dr. Stetten because he has been working in this field.

DR. SAMUELS: Coming back to Dr. Stetten's observation on this action. I think one of the dangers in the past in the application of the Michaelis-Menten concept is the failure to recognize that these enzyme reactions involve other interaction, besides the ones most easily observed. I think this is particularly brought out in the case of hexokinase, where the ADP-ATP equilibrium reaction on the enzyme affects the rate of the reaction between ATP and glucose. The hormones could affect the rate of a reaction by affecting the binding of the other components of the reaction. In that connection, does anyone know whether there have ever been any studies of the effect of insulin on the ADP-ATP equilibrium, since that would ultimately affect the whole rate of the reaction.

DR. LARDY: That reaction has been studied now as an equilibrium. It is known, of course, that the hexokinase reaction drives the phosphorylase virtually to completion, but by using isotopically related materials it is possible to show that it is a true reversible reaction. Labeled glucose-6-phosphate with P^{32} results in P^{32} incorporation into the ATP but I don't believe that people who have studied this equilibration have investigated the enzymes, or the effect of hormones on that reaction. For a long time we thought that the current effect of insulin on hexokinase might not be at the actual phosphorylation step but at the steps where glucose-6-phosphate is disposed of. The reason for that is that the hexokinase reaction is one which is regulated by negative feedback and that the product of the reaction is the powerful inhibitor of the enzyme. Glucose-6-phosphate that is produced is a very

sulin which is without effect on the glucose uptake of the isolated rat diaphragm provides an estimate of the amount of insulin present in the tissue.

These arguments are, I think, open to criticism for several reasons. The studies of Krahl and Cori showed that the diaphragm of the alloxan diabetic rat, which has a very low basal glucose uptake does not show an increased response to insulin (Krahl, M. E. and Cori, C. F.: *J. Biol. Chem., 170:* 607, 1947). Also Dr. Stadie's results suggest that the diaphragms of hypophysectomized rats, which have a high basal glucose uptake, are more sensitive to insulin than the normal rat diaphragm. That again would be the wrong way round from the point of view of the suggestion of Ottaway and Kerly. There is though some disagreement about the response to insulin of the diaphragm from the hypophysectomized rat *in vitro*, because Krahl & Park reported that this preparation showed a diminished response to insulin (Krahl, M. E. and Park, C. R.: *J. Biol. Chem., 174:* 939, 1948).

That brings me to another point. What is the measure of sensitivity to insulin in an *in vitro* system? There are three possibilities. Firstly, one could measure the minimum concentration of insulin which produces an effect. Another measure of sensitivity to insulin would be the increment of glucose uptake which one gets with increasing concentrations of insulin. The third measure of sensitivity, which has been used particularly by Krahl and his associates, is the maximum increase in glucose uptake which can be evoked by high concentrations of insulin. I think the latter measure of sensitivity is perhaps not reliable, because as Dr. Stetten has already emphasized, tissues probably have a limited capacity to utilize glucose, and if a tissue already ha a high rate of glucose utilization *in vitro*, in the absence of added insulin, then one is not going to obtain much greater glucose utilization with the addition of insulin.

I think there is one further tissue which Dr. Stadie did not discuss with respect to insulin binding, and that is the heart, which as Fisher has shown will respond to insulin *in vitro* by an increased rate of glucose utilization. Insulin binding may be a lax affair in the heart because Fisher has shown that a short period of washing with an insulin free medium will suffice to abolish the

recently obtained evidence suggesting that the reticulin fibres of muscle and spleen may bind insulin (Chayen, J. and Smith, R. H.: *Biochem. J., 58:* VIII, 1954; Ottaway J. H.: *Biochem. J., 58:* VIII, 1954). It seems, I think, unlikely that insulin bound to reticulin fibres would influence the metabolism of muscle and there may well be another site of binding at which insulin exerts its influence on metabolism.

Dr. Stadie's results suggest that the influence of insulin on the synthesis of glycogen by the isolated rat diaphragm is proportional to the amount of insulin bound by the diaphragm. We have been interested in the use of the isolated diaphragm from normal rats to assay insulin and have studied the relationship between the glucose uptake of the isolated diaphragm and the concentration of insulin in the suspending fluid. If one plots the cube root of the uptake of glucose by the diaphragm against the log concentration of insulin in the suspending fluid one can get a very good straight line. This we have confirmed in some 18 or 20 studies of regression. The log dose response line is not a constant line. The slope varies and so does the concentration of insulin at which it begins. Thus from one day to the next we have a family of straight lines which express the relationship between glucose uptake by the isolated diaphragm and the concentration of insulin in the suspending fluid (Randle, P. J.: 1956, *J. Endocrinol.,* 1956. In press). Thus the relation between insulin effect and insulin concentration in this system is not a simple and constant one.

Now it has been suggested by Ottaway and Kerly that the response of the isolated rat diaphragm to insulin is a function of its basal glucose uptake—that is its glucose uptake in the absence of insulin (Ottaway, J. H. and Kerly, M.: *J. Physiol., 123:* 534, 1954). Ottaway and Kerly found with rats fed different diets that the response of the diaphragm to insulin was inversely proportional to the basal glucose uptake. One possible interpretation of these results would be that the basal glucose uptake of the diaphragm provides a measure of the amount of insulin in the tissue and that the response to added insulin is reduced when the insulin content of the tissue is high and vice versa. If that is the case, then one could argue that the maximum concentration of added in-

But certainly brain and red blood cells do not show any action of insulin. Now, these cells contain, as far as we know, the whole list of enzymatic intermediary reactions which Dr. Stetten summarized under respiration, glycolysis, etc. Certainly they contain glucokinase, and yet insulin does not appear to effect it. Two possibilities exist to account for this specificity of action: either the enzymes are structurally different, i.e., the glucokinase of brain is a different group of proteins from the glucokinase in muscle; or if they are both the same, then insulin must act on something which is specific for muscle. The difficulty comes in this: that the transfer of the glucose across the cell wall, if you take red blood cells and compare them to muscle, seems to have the same kind of structural specificity. For instance, Willbrand has shown that glucose, galactose, l-arabinose and d-xylose in a fashion similar to what we found in muscle, penetrate and are transferred. So the transfer system may also be similar. Therefore, ultimately, we *must look for something which is really specific for those cells* which are responsive to the hormones. The other thing which I would like to bring to your attention is that hormones arose quite late, in the evolutionary scheme of things. I believe those hormones with which we are working in the mammalian field arise somewhere around the chordates, and that the enzyme systems which are familiar to all of us, seem to be similar throughout the whole biological kingdom. Intermediary metabolism of the yeast cell, the intermediary metabolism of bacteria, the intermediary metabolic scheme in muscle, seems to be based on similar enzyme reactions. The hormones came in later. In common with the nervous system, their function seems to be to relate what goes on in one tissue to what goes on in another one, because it is very difficult to get information from one cell even to the next contiguous cell. As far as we know, energy is never transferred across a cell wall. Even inside the cell, energy is probably only transferred to contiguous areas. However, as Dr Stetten pointed out, metabolites are transferred It would seem, therefore, reasonable that an important action of hormones would be the regulation of exit and entry (1). It would be a very powerful kind of integrating mechanism, and this I think we are nearer to in

[1] *Recent Prog Hormone Research, 11.343,* 1955.

insulin effect in the perfused isolated heart (Bleehen, N. M. and Fisher, R. B.: *J. biol. Chem., 123:* 260, 1954). However, that is not evidence against the view that insulin is bound by tissues on the site at which it acts, for it does not seem necessary that insulin should be bound irreversibly by tissues on which it acts. Dr. Stadie has mentioned that he could find no evidence that the kidney binds insulin. In preliminary experiments we have observed a small increase in the uptake of glucose by kidney slices *in vitro* with the addition of insulin. There again however the binding might be a loose one.

DR. LEVINE: If I may, I would like to inject a little physiology into biochemistry. Dr. Stetten's and Dr. Stadie's discussions have raised the question of the site of action of insulin. All hormones seem to show a predilection for certain tissues and certain cells. Other cells, which we know possess the same kind of intermediary metabolic scheme do not seem to be affected by the hormones. Let's take insulin as an example since I am more familiar with that. It is easy to demonstrate an "action of insulin on muscle." Whether that action really is on the isolated muscle fiber, nobody yet knows. I would like to point out that even *in vitro*, one uses a diaphragm which contains, in addition to muscle fibers, a large number of connective tissue cells. Since insulin acts upon adipose tissue, modified connective tissue, the problem arises whether this type of cell may not be the *only* type of cell that responds to insulin. Nobody has yet demonstrated an isolated muscle cell free of the sarcolemmal nuclei. This is just a side thought. But muscle responds, and we know that skeletal muscle does and cardiac muscle does It is not known whether smooth muscle does. The possibility exists that it does respond. Of the other tissues, there is the fat tissue which is connective tissue, and the mammary gland. Now the question arises whether in the mammary gland the response is on the epithelial elements, or on the connective tissue elements.

There are certain tissues which, everybody agrees, are not sensitive to the action of insulin, namely brain, and red blood cells. Despite a few experiments in which there is an indication that there is an action of insulin on the liver, the major portion of the literature seems to be against there being any action on the liver.

ically speaking, this appears to be a good thing. Otherwise the level of blood sugar could not be maintained, and the brain would suffer. There are at least two circumstances when this is changed. One circumstance is when a glucose load is given to an animal. It raises the blood sugar. This results in stimulation of insulin secretion by the pancreas, as Dr. Houssay has shown a long time ago. This insulin serves to open up the previously barred areas, the muscle and the connective tissue, and the load of glucose is stored away. The other condition which produces this effect is work. This also opens up areas of muscle and stimulates hepatic glucose production. If the work is excessive, it leads to hypoglycemia. In other words, insulin, and perhaps other hormones, are *not* regulators of unicellular metabolism. Rather, they are integrators in a metazoal organism. It would, therefore, be reasonable to expect that some of the hormones in addition to regulating metabolism, act upon the boundary areas between cells and the transported glucose. It doesn't require a knowledge of mechanism; it simply requires a site of activity.

DR. BEST: I have only one or two small points. Dr. Poulsen, who works with Dr. Hagerdorn, has made a study of intraportally administered insulin and finds a delayed action as compared to administration by other routes. Dr. Poulsen's suggestion is that the liver fixes insulin in some way and liberates it gradually, and thus you get a more delayed action.

Someone will perhaps refresh my memory, but I thought that in Dr. Hasting's laboratory when insulin was given over a period of time to the intact animal a very definite effect on either glycogen formation or glucose uptake was secured in liver slices.

DR. LEVINE: Yes, Dr. Best, but Dr. Hastings points out that both his and Chaikoff's experiments seem to indicate that the effect is on muscle.

DR. SAMUELS: In regard to the comments Dr. Levine was making on the site of action of hormones· this, of course, is something that has interested many endocrinologists who think in terms of reactions. I was always inclined to agree with the group that

the action of insulin than in any other hormone—and this is by no means again an original notion. This is a notion very forcibly expressed years ago by Faber on the basis of his study of what he called "physiological permeability in the cell." He actually related it to the disturbance in diabetes. One of his men in Kino, found that red blood cells for various species differed in the kinds of sugars they could take up. He speculated at the end of that paper, and this was in 1914: "Is it possible," he said, "that the disturbance in diabetes mellitus is a loss of the physiological permeability of certain cells to glucose and that the (then unknown) hormone serves to regulate this physiological permeability (2)?"

The other thing to which I wanted to draw your attention, came out of the work we did on insulin and, at the same time, on the effects of work (3), (4). As you know, muscular work requires that more sugar get into the muscle cell, and much more sugar is used. Since we found that the transfer of sugar from the outside to the inside of the cell was promoted by insulin, and that there was ordinarily a barrier to free entry, work would also have to be expended in order for free entry to occur. We found this to be so. How this is regulated is, of course, not known. I would like to point out the possible functional significance of this. Let's take insulin and work. Under ordinary circumstances in the mammalian organism when no food is being actively absorbed, the liver produces sugars from its stores, and from protein and other materials. In the dog, the hepatic production of glucose has been roughly calculated to be somewhere in the neighborhood of 150 to 200 milligrams of sugar per kilo per hour. Dr. Crandall, I think, has figures of this order. When one then compares this figure to the amount of glucose utilized after rest by the central nervous system and the heart, the two together account for somewhere between 80 to 85% of the total hepatic production. This sugar is, however, circulating all over the body. A great mass of cells in the central nervous system and the brain, seem to be prevented from taking up their sugar during those circumstances. Teleolog-

[2] HOBER, R. *Biochem. A.*, 60:253, 1914.

[3] GOLDSTEIN, M. S., MULLICK, V., HUDDLESTUN, B AND LEVINE, R *Am. J Physiol.*, 173:212, 1953.

[4] LEVINE, R. AND GOLDSTEIN, M. S., *Brookhaven Symp. in Biol*, 5:73, 1952.

sumption. In other words, there is a local effect of work in the contractile tissues themselves which accounts for the oxygen consumption; a remote effect of work, probably humeral, which is exerted on the rest of the body by something coming from the exercising muscle which has properties similar to insulin in facilitating the entry of sugars even into cells which are not working.

DR. LARDY: It seems inevitable that the discussion rests around insulin, so I will ask a question I was going to hold for Sunday afternoon. That is concerning an experiment you did on the effect of cortisone on galactose penetration. I don't remember exactly but I believe it was one of your first papers in 1950 on the problem, which showed that previous cortisone administration abolishes the effect of insulin upon glucose, but does not abolish the effect of insulin upon entry of galactose into muscle. Is that correct?

DR. LEVINE: That is essentially correct. We gave a dog a series of injections of cortisone of 25 milligrams a day for several days, and then tested his insulin sensitivity in regard to glucose. He became very insulin insensitive. We then eviscerated that dog and gave him galactose. It disappeared as though cortisone had not been given. I think you are referring to the contradiction; why did it abolish the glucose effect but not the galactose effect. I think the reason is simply this: cortisone does not oppose insulin in the periphery That is, if you have a peripheral cell and this be the site of insulin action . . . some patch represents the transfer system Insulin comes along, and there is facilitation of entry. How are we going to know? This experiment, using galactose, demonstrates to us at least, *that cortisone does not settle on the same* patch to interfere with this action. How, then, does it interfere with the drop in blood sugar in the intact animal? We think that it does so by increasing gluconeogenesis in the liver to such an extent that the action of insulin upon the blood sugar is not seen. If one were to give a larger amount of insulin, you would see it. The same effect would be achieved by giving insulin and sugar together. The word "insulin antagonist," we think, has been used rather loosely. Any agent which abolishes *the effect of hypogly-*

thought that perhaps these things might be membrane reactions, since two types of compounds that might be thought of as acting on surfaces, proteins (or very large polypeptides) and steroids make up most of the hormones. But I have begun to think otherwise for two reasons. First, the evidence seems fairly good now that ACTH can act upon *in vitro* systems in which the cells are disintegrated. Second, the recent observation of Villee regarding the apparent influence of estrogens on a common energy system in an homogenate of placenta. Now if the hormones act inside the cell, I always thought that they acted on enzymic reactions peculiar to particular cells, and that seeking for their action in a generally occurring enzymic system was rather a waste of time. But if Villee's observations hold up, we must face the fact that a term such as isocitric dehydrogenase is generic, and not the name of a particular molecule. I believe Dr. Smith at Utah feels that he has split off a good part of the papain molecule without affecting the molar rate of reaction. So these enzymes can have tails on them which may affect their reaction with many things, which may not be specifically related to the site of the major reaction but which could affect energy relationships within the molecule. So I think we should remain open to the idea that the action of the hormones can be intracellular, and might even be on some common reactions, the particular molecules catalyzing the reactions within the specific cell being slightly different.

DR. WICK: Dr. Levine, are insulin action and the work effect comparable? Insulin has no effect on oxygen uptake. I think we all agree on that. But how about work? Work has a tremendous effect on oxygen consumption; so can you properly compare the work effect and insulin effect upon glucose uptake?

DR. LEVINE: Well, if I may venture, I will say this. Insulin and work have a similarity in the fact that they both facilitate transfer of the sugar from the circulation into the particular cells. But there the similarity ends because work has another effect, and that is a purely local effect in the working muscle, where, by means of utilizing a tremendous amount of ATP, it always pulls the reaction of respiration with it and therefore increases oxygen con-

Dr. Houssay: Dr. Levine has reminded us that we have to pay much attention to the role of hormones sent by one organ and transported by the blood as regulators of the function of another organ or tissue. Another accessory point to be remembered is that the alloxan diabetic animal is not completely deprived of insulin. It would be much better if the biochemists, instead of using the alloxan diabetic animal would begin using totally depancreatized rats. These animals are not easy to prepare, but Dr. Robert Scow has studied the techniques for the operation and management of these rats and has used them. The animals are quite different from the alloxan-diabetic animal.

Another point to be remembered is that hormones have regulatory action on preexisting phenomena; they do not produce new functions. For instance, glucose utilization can be observed in organisms that have no insulin. But the regulatory action of insulin is so important that when it is not present, severe and fatal diabetes is produced in many species.

Another question very important in physiology is that in the whole organism one hormone never works alone. In every case, the action of one hormone is connected to that of other hormones; the normal action of one hormone is related to the balance of hormones present. If we study any function we find that it does not depend on one hormone, but on a balance between hormones acting together or in a consecutive way. For instance, the mammary gland develops completely by action of estrogen, progesterone, prolactin and somatotrophin and not by one of these hormones alone. The endometrial changes are due to the interplay of FSH, LH, estrogens and progesterone.

The carbohydrate metabolism is normally regulated by a balance of hormones of the pancreas, pituitary, adrenal, thyroid, and in some cases, gonadal hormones. The action of one hormone or gland is modified by the presence of another hormone or gland. Without the adrenal there is striking diminution of some actions of somatotrophin or thyroxin. In the organism we have always interaction between hormones, but never is one hormone acting completely alone That is an important concept to be kept in mind when working in endocrinology.

cemia in the intact animal was called an insulin antagonist. The implication was that it acted at the same site. In order to abolish the hypoglycemia, it need not act at the same site. This is all we were interested in showing. This has also been shown with at least one batch of growth hormone.

DR. LARDY: May I ask another question? I am trying to get away from insulin, in reference to Dr. Stetten's concept of the Mc-Calebs process. There is a rationale for 1/V versus 1/S in the Line-weaver-Burke. I would like to ask Dr. Randle what the rationale is for plotting the cube root of the glucose uptake?

DR. RANDLE: There is no rationale. This is a purely empirical procedure. It just so happens that it gives the best straight line relationship compared to log of dose. I would not put any interpretation on it. It was purely empirical.

DR. LARDY: Using cube roots or even higher roots is one way to get variable data to come much closer together; yet it will not make a straight line out of data which are completely at random, so there must be some fundamental reason for it.

DR. KINSELL: Do you have some thoughts as to the possible fundamental reasons, Dr. Lardy?

DR. LARDY: None whatever.

DR. SAMUELS: I think there is one thing that has to be thought of in applying mathematics to a hormonally controlled system: that is that one is usually measuring the reaction which has been catalyzed by the enzyme system; this in turn, has been catalytically affected by the hormone. There is, therefore, an added complexity to the mathematics involved, and unless one can create the condition where the other reaction becomes O order, one must know all the components of that system, the co-factors as well as the major substrates involved. That may be the reason why complications arise when applying enzyme kinetics to a hormonal system.

DR. ASTWOOD: I would like to hear more about the influence of cortical hormones on the utilization of carbohydrate. Does the adrenally deficient animal use carbohydrate more quickly than does the normal animal, and is Dr. Levine correct, that cortisone does not in any sense inhibit the action of insulin?

DR. STETTEN: In experiments conducted several years ago in collaboration with Dr. Dwight Ingle we studied, in the intact anesthetized animal, the dilution of radioactive glucose administered at a constant rate intravenously. We compared the alloxan diabetic rat and the rat who had received, by current standards, rather astronomic doses of cortisone prior to the experiment. From the data secured, notably the specific activity of the urinary glucose collected and of the carbon dioxide excreted, we were able to calculate two sets of quantities. One was total glucose synthesis in the animal from non-isotopic precursor, and the other was the contribution of body glucose to the carbon dioxide excreted. In the alloxanized animal there was a striking diminution as compared with the normal animal in the contribution of glucose to carbon dioxide. This fell by a matter of 60% from a normal level. There was some evidence of increased glucose production in these animals, as measured by the dilution of the isotopic injected glucose, approximately a doubling of the glucogenic rate. In cortisone-treated animals, the glucogenic rate as measured by dilution of infused glucose, went up by a factor of six or seven fold over the normal levels. There was no evidence of change from normal in the over-all sequence from injected glucose to carbon dioxide. From this we concluded that the hyperglycemia, the glycosuria which Ingle consistently gets in rats treated with cortisone by this method, was predominantly a consequence of excessive production of glucose from precursors not identified in this experiment, and that there was no severe impairment in the over-all conversion of glucose to carbon dioxide. In the alloxanized rats there was impairment of the total oxidation. There was also some increase in glucose production, but we couldn't attribute this necessarily to insulin since these alloxanized rats in Ingle's colony constantly had hypertrophic adrenals as compared with the normals, and we thought that it might be a secondary adrenocortical effect that we were observing.

DR. GROSS: I would like to ask how Professor Houssay's remarks apply to the mechanism of metamorphosis produced by hormones?

DR. HOUSSAY: This is an effect of the pituitary gland on the thyroid gland secretion that produces metamorphosis. But there are very complicated facts about metamorphosis. For instance in the Base Institute of Calcutta, Dr. Nandi showed me that penicillin abolished metamorphosis of tadpoles, but adding vitamin B_{12} or thyroxin, produced metamorphosis. We know also that metamorphosis is produced by iodine in tadpoles without thyroid. In this case it is not the thyroid that provokes metamorphosis, but probably the thyroxine or similar substance produced by iodine acting on the proteins of the thyroidless tadpole.

DR. GROSS: Well, of course there is a complication there because the skin of the amphibian would take up iodine and convert it into protein bound iodine, and so one is not dealing with a single site of possible hormone or possible metamorphosis producing substance.

DR. HOUSSAY: But what is the physiological phenomenon? The sequence of events is: first, pituitary, second, thyroid, and third the action of the iodine compound produced. There are three steps. In the animal without pituitary there is no metamorphosis. The physiological phenomena is due at least to the action of two glands, acting one after the other.

DR. ROBERTS: It seems to me that metamorphosis can be considered as much a problem of regulation as any other type of physiological action the hormones may exert. Metamorphosis, is, of course, the process of differentiation of particular cells. It is easy to conceive that a certain hormone may have a regulatory influence on very specialized cells which permits those cells to utilize substrate more effectively and undergo differentiation. Whether this occurs via the effects of the hormone on enzyme systems would not be critical. In either instance the final action might be similar to any other hormonal action.

DR. LEVINE: The ability of the adrenalectomized animal to do muscular work doesn't seem to be related to any metabolic impairment. A muscle taken from an adrenalectomized animal (after the animal had been fatigued by work *in vivo*,) *in vitro* has normal, contractible ability. The *in vivo* fatigue which is relieved by C-11 steroids seems to be due to a circulatory effect which follows the fall in blood pressure upon exercise of the adrenalectomized animal. In other words, the effect of the oxysteroids, seems to be exerted on the peripheral vascular system rather than on the energy apparatus of muscle (5) (6) (7).

DR. KINSELL: Would you think that perhaps the sodium-potassium relationship might be involved?

DR. LEVINE: I don't think so, because desoxycorticosterone does not prevent muscle fatigue.

DR. HOUSSAY· About the effects of cortisone on carbohydrate metabolism. First observed is the great resistance to insulin. The tolerance curve of the treated animal is that of the diabetic organism At the beginning of treatment that is very clear in the human being, but after some time there is development of better tolerance as Bastenic has found. In spite of having hyperglycemia and transitory diabetes it is not possible to maintain the high level of hyperglycemia in rabbits, rats and guinea pigs. They don't develop permanent diabetes, in spite of their having some lesions in the beta cells of the islets of Langerhans in the pancreas. Normal dogs and cats are very insensitive, but reducing surgically the pancreas (about to 15 to 20%), in these animals with normal glycemia it is possible to produce temporary (corticoid) diabetes or permanent (metacorticoid) diabetes, especially with compound F, cortisone, prednisone, prednisolone or fluor-hydrocortisone. There is at the beginning hypertrophy or hyperplasia of the islets, and probably an increase of insulin secretion, but secondarily there are lesions in the islets of Langerhans, probably a decrease of secretion of insulin, and metacorticoid diabetes is produced.

[5] RAMEY, E R, GOLDSTEIN, M. S. AND LEVINE, R *Am. J. Physiol*, *162*:10, 1950.
[6] GOLDSTEIN, M. S, RAMEY, E. R AND LEVINE, R: *Am J. Physiol*, *163*:561, 1950.
[7] RAMEY, E, GOLDSTEIN, M S. AND LEVINE, R: *Am. J. Physiol*, *165*:450, 1950.

DR. ASTWOOD: What about the older experiments showing that carbohydrate stores disappear more rapidly in the absence of the adrenal cortex?

DR. LEVINE: It was shown by Jane Russell that in the adrenalectomized animal the slope of fall of sugar was a function of the degree of shock in the animal, so that the better maintained the adrenalectomized animal was, the more nearly the drop in glucose of the hepatectomized adrenalectomized preparation approached that of the hepatectomized, normal preparation.

DR. KINSELL: In the light of the immediately preceding remarks, do you feel that one may entertain the concept that adrenocortical activity, instead of being anti-insulin, as it has been so often designated, is rather something which increases the *demand* for insulin for total metabolic activity?

DR. ASTWOOD: Increasing the demand for insulin in the sense that you have more sugar produced that has to be handled. From what Dr. Stetten and Dr. Levine have just said, that certainly seems to be the case. I can recall a statement by Ingle in a review in 1943, that there must be something more than increased glyconeogenesis to account for so-called steroid diabetes. I think that he felt at that time that there was something interfering with the utilization of carbohydrate when large doses of corticoids were given.

DR. WICK: We have compared sugar disappearance and oxidation in eviscerated and nephrectomized rabbits with animals which were eviscerated, nephrectomized and adrenalectomized. We got no differences whatsoever. The adrenals don't seem to play a measureable role in the muscle utilization of sugar under these conditions. (*Ann. New York Acad. Sci.*, *54*:684-692, 1951.)

DR. LARDY: There is another aspect of that however. In Dr. Ingle's work where there is a constant glucose infusion there is still a tremendous effect of adrenocortical hormone on the ability of muscle to do work. It would seem that under such conditions where neoglucogenesis is ruled out, there must be an effect on muscle energy production.

oids and other hormones seem to antagonize, if one may use this term loosely, the action of insulin on the entry of sugar. In *in vitro* experiments with diaphragm, cortical hormones have been added to the medium and the uptake of sugar or glycogen synthesis was found to be diminished. Glycogen synthesis as a criterion of insulin action is not wholly dependable because there are sequences of reactions involved after the entry of sugar. But there are some data on glucose uptake. The amount of the steroid needed to demonstrate an effect is incredibly high and some steroids which *in vivo* do not act on carbohydrate metabolism seem to exert an action *in vitro*. For example, desoxycorticosterone. Epinephrine has been reported to inhibit sugar uptake in muscle. This has always been a controversial problem both *in vivo* and *in vitro*. At certain dose levels of epinephrine in the medium, there seems to be some effect upon the uptake of glucose. Sutherland thinks that it is purely secondary to an increased concentration of glucose-6-phosphate which secondarily inhibits hexokinase. But that has not been worked out, and I should like to hear it discussed.

DR. STETTEN: I think one of the problems in relation to antagonism depends in part upon how myopic one is. Insulin and glucagon have been described as antagonists largely on the basis of mutually reciprocal effects on the level of blood glucose. It has always seemed to me that the concentration of glucose in the blood is relatively unimportant compared to what is happening in the tissues. From the muscle's point of view, I venture to suggest that insulin and glucagon are synergists in the sense that both of them supply or forward nutrients to the muscle, the one by facilitating breakdown of liver glycogen and increasing the blood glucose concentration, the other by making this same glucose more available to the muscles by one means or another. Insulin and glucagon thus are synergistic in the same sense that a glucose infusion is synergistic with insulin.

I would like to extend the question, which is perhaps a little unfair in the absence of Dr. Sutherland or other representatives of this group. I think it is quite convincingly established that epinephrine, as well as glucagon will increase the activity of phosphorylase which has been partially inactivated in liver. Epineph-

DR. RANDLE: Welt and Wilhelmi have obtained evidence which suggests that corticotropin or cortisone inhibit the conversion of glucose to fat (Welt, I. D. and Wilhelmi, A. E.: *Yale, J. Biol. Med.*, 23:99, 1950). In that particular instance there is evidence of antagonism between insulin and adrenal steroids. Insulin and adrenal steroids also have opposing actions with respect to protein metabolism, at any rate *in vivo*, though their actions are not of course, necessarily opposed at the same point in protein metabolism. We do not know their sites of action on protein metabolism.

DR. ROBERTS: The antagonistic effect of the adrenocortical steroids in protein metabolism which Dr. Randle referred to would appear to be capable of inhibition by any procedure which supplies the energy-yielding products of protein metabolism. Thus, the protein catabolic effect of adrenocortical steroids can be prevented by infusion with amino acids or with glucose, as Engel has shown. This need not be a true antagonistic action since the corticosteroids and the infused metabolites might have their actions on different phases of protein metabolism.

DR. RANDLE. If one stops to think about insulin antagonists, there are several possible mechanisms of action. They could accelerate insulin destruction or prevent insulin from reaching its site of action, as for example by interfering with binding. They could act by opposing the action of insulin directly—that is by slowing whatever process in carbohydrate metabolism it is that insulin accelerates, or alternatively they might antagonize the action of insulin indirectly by restraining some other reaction in carbohydrate metabolism upon which insulin has no action, but which is essential to the action of insulin. Or alternatively they might act by reducing the availability of some substrate or co-factor essential for the action of insulin. Direct antagonism between insulin and other hormones at the site of action of insulin cannot be demonstrated until the mechanism of insulin action is clearly understood.

DR. LEVINE: There are some indications in the literature which I would very much like to bring up for discussion, that adrenal ster-

liver slice *in vitro*, while phosphorylase is being inactivated, goes along at a fairly constant rate for 1½ to 2 hours.

DR. LARDY: I think there are two problems, one in muscle and one in liver. According to Sutherland's data, the phosphorylase is in an inactive form in fresh liver slices and as you point out, becomes inactivated during the 20 to 40 minute incubation period. If you incubate with epinephrine during that 20 minutes, you prevent that inactivation, or if you add epinephrine after the 20 minutes period within a period of I think, three minutes, the shortest time Sutherland has used, the enzyme is reactivated. I think that's a real phenomenon and one that we have to take into account. It can't be the primary effect of the hormone because of the very sudden creation of free glucose. As far as the effect on rate of liberation of glucose is concerned, there may be the matter of another enzyme such as the glucose-6-phosphatase which is the rate limiting one. Maybe the concentration of phosphorylase is not rate limiting. In muscle, however, it used to be thought that fresh muscle contained phosphorylase A, and that on heating the muscle it was converted to phosphorylase B which is inactive except on the addition of the adenylic acid. More recently, however, Drs. Cori and Carpenter, and especially Krebs and Fisher at Seattle have shown that fresh muscle probably contains phosphorylase B, and that it is activated by ATP and the extract when the phosphorylase is isolated. Dr. Cori has recently taken up the study of this phenomenon in animals under various treatments to find out what factors regulate the interconversion of phosphorylase A and B. But I think in muscle, as well as in liver, we have to consider the concentration of inorganic phosphate that is available to the phosphorylase equilibrium. We could certainly never get the glycogen synthesized in the first place if there were a higher concentration of inorganic phosphate than you would predict would synthesize glycogen. You have to have one of your components rate limit in low amount in order to get the reaction to go in that direction in the first place. One possibility is that you hold the glycogen into some entirely different form. It is known that there are two different forms of glycogen in most tissue, one which is soluble in trichloracetic and one which is not. It is conceivable

rine does this in muscle also. Phosphorylase, however, catalyzes a readily reversible reaction, reversible not only in the animal but in the test tube, yet the effects observed for epinephrine, and so far as I am aware, for glucagon, have always been in one direction. It has seemed to me that Dr. Sutherland's results are at best a partial explanation of what is observed in the intact animal; i.e., in the well nourished animal, epinephrine always, as far as I am aware lowers the liver glycogen and enhances the blood glucose concentration. I am not clear as to how by enhancing the abundance of an enzyme that catalyzes a reversible reaction, one can in the animal always be sure that the reaction is always stimulated in one direction.

DR. LARDY: I have exactly the same convictions as Dr. Stetten. I think these conclusions are unavoidable, if you consider the reactions that Dr. Stetten has spoken about earlier. One is dealing with an equilibrium reaction, yet the effect is always in one direction. Therefore, the result must be some other primary effect of epinephrine, and the effect on phosphorylase activation must be a secondary one.

DR. LEVINE: Dr. Lardy, would you consider adding to your manuscript an answer to the following question related to epinephrine action. Dr. Sutherland's work is excellently conceived experimentally. The liver slice is incubated at 37° in the Warburg for 30 minutes or more. The active phosphorylase is then estimated by grinding the slice up and testing it. Phosphorylase activity falls during incubation unless epinephrine is present. This is a reactivation of inactivated phosphorylase. However, if you give epinephrine to an animal, within a very few seconds the liver glycogen begins to break down and hyperglycemia occurs. *In vitro,* one never sees an increase in the initial amount of phosphorylase, only a reactivation of a drop in phosphorylase. Is this because the phosphorylase *in vivo* is kept inactive and the epinephrine serves to reactivate it? If, in the absence of epinephrine the phosphorylase level falls when one incubates a liver slice, one should get less and less sugar liberated with time, so that the curve should flatten out very quickly. It does not. The release of sugar from a

TABLE B

RELATIVE MOL. WTS OF DEXTRINS

Sample	*Light Scattering*		*Enz. Deg. Rel. Mol. Wt.*
	Mol. Wts.	*RMW*	
Glyc. (L-TCA)	25.7×10^6	100	100
Dex. (Phos.)	20.1×10^6	78	78
Dex. (β-am.)	12.1×10^6	47	50
Glyc (L-TCA)	13.8×10^6	100	100
Dex. (β-am.)	8.2×10^6	59	55
Glyc. (M-KOH)	$3\ 5\times10^6$	100	100
Dex. (β-am)	2.4×10^6	68	59

from the analysis of the small fragments produced enzymatically, and the turbidometric molecular weight determinations. At least light scattering which, unfortunately gives us a weight average molecular weight, rather than a number average molecular weight, does give fair agreement with the expected decrease in molecular weight of enzyme degradation. (Table 13).

In Table C is recorded the effect of alkali on the molecular weight In these cases the tissue is divided and the glycogen is extracted with, trichloracetic acid from one portion, while the remainder is treated with alkali. When we treat glycogen with alkali, we get molecular weights in accord with those recorded by other workers who used a similar method of estimating molecular weight of alkali-extracted glycogen. When we take a sample of trichloracetic-extracted glycogen and boil it with 30% KOH, the

TABLE C

COMPARISON OF MOL WTS OF GLYCOGENS SECURED BY DIFFERENT METHODS

Source of Glycogen	*Molecular Weight*	
	TCA Method	*KOH*
Rabbit Liver	$45\ 2\times10^6$	$2\ 7\times10^6$
Rabbit Muscle	$11\ 9\times10^6$	$3\ 1\times10^6$
Rat Muscle	$43\ 8\times10^6$	$6\ 1\times10^6$

that as glycogen is synthesized a portion of it is converted to a form which is not available to phosphorylase. And that might be one site for epinephrine to act.

DR. STETTEN: We recently have been studying the metabolic inhomogeneity of glycogen in muscle and liver. Recently Dr. M. R. Stetten and I have studied the glycogen derived from rat and rabbit tissues with respect to the distribution of molecular sizes. We have been using the method of light scattering to estimate the molecular weights of glycogen. We believe this method will give us true relative molecular weights. You will note the molecular weights in Table A are considerably larger than those of the

TABLE A

REPLICATE MOL. WT. DETERMINATIONS BY LIGHT SCATTERING

Sample	Mol Wt.
Rat Liver Glyc—TCA	$70\ 8 \times 10^6$ 70.7×10^6
Rat Liver Glyc—TCA	$45\ 2 \times 10^6$ 41.4×10^6 41.1×10^6
Rabbit Muscle Glycogen—KOH	$3\ 1 \times 10^6$ $3\ 0 \times 10^6$

common proteins They may range from 2 million up to 100 million in size. We have found, as some, but not all other investigators have found, that glycogen which has been exposed to alkali invariably has a lower molecular weight than glycogen which has been extracted from tissues with the cold trichloracetic acid. Table A gives some indication of the reproducibility of the method, these being duplicate and triplicate analyses. The basis for assuming that the molecular weight method gives reasonably dependable values, is that one can take glycogen and compare its molecular weight with dextrins derived from this glycogen, either by phosphorylase or beta-amylase degradation. One finds reasonably good concordance between the molecular weights calculated

fasted for varying periods of time and glycogen was isolated from the liver. We sought for correlation between the quantity of glycogen remaining in the liver and its molecular weight. The argument here is very simple. We considered the possibility that the changes in quantity of glycogen were not a reflection of the changes in the number of molecules but rather a change in mean molecular weight. The polydispersity of glycogen has been well established, and as I will show you later, within one sample of glycogen we can find molecules varying by a factor of 20 in molecular weight. It was appealing therefore to consider that glycogen depletion and repletion was a matter of change in molecular size of the glycogen molecules rather than change in number. One reason for considering this was that in the system of enzymes described chiefly in Cori's laboratory no device has been established for the replication or the multiplication of glycogen molecules, only for their increase and their decrease in mass. We viewed the possibility that the number of glycogen molecules was constant during such a fasting experiment. What we actually found is given in Table D. When we subjected these numbers to statistical analysis, it turned out that there was a barely significant negative correlation between molecular weight, as measured by light scattering, and the quantity of glycogen. In other words, in those livers in which the quantity of glycogen was small it had a slightly greater molecular weight on the average than it did in those samples in which the glycogen quantity was large. We therefore undertook to fractionate glycogen and this turned out to be quite simple. The first method was fractional precipitation with

TABLE E

DISTRIBUTION OF RADIOACTIVITY IN RAT LIVER GLYCOGEN 6 HOURS AFTER I P GLUCOSE-C^{14}

Sample	*Quant Mg*	*Mol Wt* $\times 10^{-6}$	*Sp Act cpm/mAC*
Glycogen	74 0	25 7	47 7
Fraction 1	8 6	45 9	40.1
2	32 0	26 3	46 5
3	15.8	21 3	47.5
4	16.7	9 1	50.1

molecular weight in general falls to a level of about 2 to 4 million, and then falls very slowly thereafter, suggesting that in a sense there is a limit size of alkali digestion.

DR. LARDY: When you make KOH glycogen, you take the total tissue or do you extract the thing with TCA and then treat that with KOH?

DR. STETTEN: We take total tissue. In contrast to others we find very little difference in the yield of glycogen by the two methods of extraction. We disagree with reports in the literature that one extraction with trichloracetic acid gets all the glycogen out. In our hands it certainly doesn't. I don't know why this is, but on repeated extractions we always get more glycogen on second, third and even fourth extraction, and if we repeatedly extract with cold trichloracetic acid we recover essentially as much as is secured with hot alkali. In order to study whether glycogens of differing molecular sizes enter metabolic reactions at different rates within tissue, a simple experiment was conducted in which animals were

TABLE D

CORRELATION OF QUANTITY RECOVERED WITH MOLECULAR WEIGHT OF LIVER GLYCOGEN SAMPLES

Glycogen samples were isolated by extraction of the livers of rats with cold trichloracetic acid solutions

Quantity of Liver Glycogen	*Time After Last Meal*	*Molecular Weight*
mg	hrs	
862	0	$34\ 9 \times 10^{6}$
750	0	$13\ 8 \times 10^{6}$
607	0	$15\ 4 \times 10^{6}$
495	4	$46\ 4 \times 10^{6}$
337	4	$42\ 5 \times 10^{6}$
264	6	$70\ 8 \times 10^{6}$
125	2	52.5×10^{6}
97	12	64.4×10^{6}
57	15	47.9×10^{6}
43	18	$64\ 5 \times 10^{6}$
42	18	$57\ 1 \times 10^{6}$
23	18	79.5×10^{6}
5	18	$44\ 2 \times 10^{6}$

associated pituitary factor? This will receive more attention later by you and others I am sure.

DR. RANDLE: I have a few comments at this stage. What I shall refer to as the growth hormone can have apparently opposing actions on carbohydrate metabolism. In acute experiments, growth hormone *in vivo* can, like insulin, cause a fall of blood sugar level and an increase in the utilization of glucose by muscle. This insulin-like action of growth hormone occurs in the absence of the pancreas. In more chronic experiments growth hormone is an insulin antagonist with respect to carbohydrate and fat metabolism. Thus growth hormone depresses glucose utilization by muscle, diminishes the hypoglycemic effectiveness of insulin and inhibits the conversion of glucose to fat. The mechanism of these anti-insulin actions of growth hormone is not known. Insulin and growth hormone appear to have synergistic effects in so far as protein anabolism is concerned.

Growth hormone, in the form in which it is extracted from the pituitary gland, does not antagonize the action of insulin upon the metabolism of isolated tissues. The only definite action of growth hormone upon the metabolism of isolated tissues is an insulin-like action—that is it increases the uptake of glucose by certain tissues (diaphragm and mammary gland). The observations of Park and Bornstein suggest that growth hormone may be transformed *in vivo* to another molecule which will antagonize, *in vitro*, the action of insulin upon the uptake of glucose by muscle. Our own observations would agree with this suggestion. The mechanism of the anti-insulin effect of this possible transformation product of growth hormone is unknown. I shall not say more at this stage as I hope to discuss this aspect of growth hormone action in detail in my presentation tomorrow.

DR. BEST: If I can keep away from insulin, I might talk for a moment about glucagon—not very far away I will have to admit. The chief contributor to this field, as you all know, was until recently Burger in Germany. His conception is that glucagon is a synergist of insulin. There have been experiments by De Duve and Vuylsteke *showing that you can antidote insulin by continuous* injections of glucagon. Dr. Wick and his colleagues showed

ethanol. A second method of fractionation was fractional centrifugation in the quantitative Spinco apparatus. C^{14} glucose was injected into animals under various circumstances and glycogen was recovered from liver and muscle. Its molecular weight was determined and its specific activity was measured. It was then subjected to fractionation, each fraction undoubtedly still highly polydispersed. It will be noted that as the molecular weight of successive fractions of liver glycogen decreased, the specific activity appeared to increase slightly and this was repeatedly and consistently observed. In muscle samples something quite different

TABLE F

DISTRIBUTION OF RADIOACTIVITY IN RAT MUSCLE GLYCOGEN 3 HOURS AFTER I P. GLUCOSE-C^{14}

Sample	*Quant. Mg*	*Mol. Wt. $\times 10^{-6}$*	*Sp. Act. cpm/mAC*
Glycogen	99.3	20.5	227
Fraction 1	12.0	52 2	310
2	43 6	15.5	207
3	9 8	12.9	203
4	21 0	6.2	140

happens. Here by far the highest specific activity is associated with the fraction containing molecules of the highest molecular weight. Again this was a repeated and consistent finding. It would appear that in the muscle the entry of glucose into glycogen seems to be predominantly into the larger molecules of the population, whereas in the liver it seems to go somewhat more readily with the smaller molecules in the population. I mention this because it seems to us, not that there are two species of glycogen in these tissues, as some have supposed, but that there is an essentially infinite variety of glycogens in this completely polydisperse mixture. This is fairly characteristic of polysaccharides, in contrast to proteins.

DR. LARDY: That is a very important series of experiments.

DR. KINSELL: Dr. Randle, do you wish to make any preliminary comments regarding anti-insulin effects of the growth-hormone-

couple of years giving adrenalin in oil to dogs without getting anything comparable to this glucagon diabetes in rats.

DR. SAMUELS: I wanted to raise two questions. Dr. Stetten, regarding the character of the glycogen within the liver cell as compared with that in the muscle where you don't find such definite polydispersion: *first*, have you made any attempt to eliminate particulate glycogen, and then see how polydispersed is the soluble glycogen within the liver cell; *second*, in making the TCA extracts, have you centrifuged these at such high speeds that any particulates would be eliminated?

DR. STETTEN: After we became aware of the molecular size we avoided all filtration procedures and all high speed centrifugations in the preparation of all our glycogen samples. We found, for instance, in the application of the method of light scatterings, where dust is a serious problem, that we could not subject glycogen solutions to the usual filtration procedures. We found that by repeated filtration through the fine grade of sintered glass funnel, we could quantitatively remove all glycogen in solution. We therefore had to operate with the maximum exclusion of dust that we were capable of obtaining Fortunately, since glycogen has an enormous molecular weight, the error introduced by dust becomes less important than it does when treating with materials that have less intrinsic turbidity than glycogen does. We did, of course, free our solvents of dust by filtration. We make no claim that what we get out is native glycogen. In fact, it is questionable to me whether by any methods at present available one can be sure of the native state of a polydispersed material which has no peculiar endocrinological or pharmacological action. We know that on prolonged exposure to trichloracetic acid the molecular weight of glycogen falls, and undoubtedly falls somewhat even during our mild method of isolation. Given a sample which is purported to be native glycogen I don't know how one can establish that this is the case.

DR. SAMUELS: Only perhaps in this way: you can study the liver which has been depleted of glycogen. The amount of the cytologi-

some interference with the utilization of sugar after insulin in eviscerated animals. You might like to say something about the extent of this inhibition Dr. Wick. But in general there have been several series of experiments—some reported recently by Root of the Eli Lilly Company and by Foa of Chicago which have not revealed any diabetogenic effects of glucagon. But if you give glucagon in large doses as Dr. Salter, Dr. Davidson and I have done recently, you get an intense diabetic action of glucagon in a resistant species. Rats become very diabetic within a few hours and their blood sugar may go to 1000 milligrams per cent. They may put out a great deal of sugar in the urine—up to 10 grams. Glucagon under these conditions is much more diabetigenic than growth hormone in the white rat. The dose of glucagon is very large although it has been given more physiologically, that is, over a long period of time. There is a 100% increase in nitrogen excretion in these rats. We haven't kept them going long enough yet to make them permanently diabetic but this is being attempted. They have exhibited a prolonged diabetes. This may not be a physiological action of glucagon. In dogs glucagon causes glucosuria and hyperglycemia. In the one animal that we studied the insulin content of the pancreas was one-tenth of the normal after four or five days on glucagon. There are extensive histological lesions in the pancreas of the glucagon-treated rats.

DR. KINSELL: Does this happen in the absence of the adrenals?

DR. BEST: It has only been done in intact animals. The curious thing about the glucagon injected animals is that they lose their appetites. There is no evidence of other toxic effects of the material in this dosage, but they don't eat and the experiments, when diabetes was produced, were done with force-fed animals.

DR. LEVINE: I would like to ask Dr. Best, are similar experiments planned or being done, using adrenalin?

DR. BEST: I have tried very hard over many years to get permanent diabetes with adrenalin and have failed completely. Two very energetic Ph.D. students Dr. Adams and Fraser worked for a

II

HORMONAL REGULATION OF ENZYMATIC ACTIVITY

By Henry A. Lardy

This subject of hormonal regulation of the activity of enzymes is rather flexible. We could discuss all the aspects of hormonal effects on enzymatic action *in vitro* and *in vivo*, but that would take a long, long time. If we were to limit ourselves to the responses that represent "Primary Effects" of hormones on enzymes I'm afraid that we might have nothing to talk about at all. We shall have to strike something of a happy medium. I would prefer to take just a few examples of enzymes that are known to be affected by hormones and use these as a means of opening the program. The direction of the discussion can proceed as you wish.

We have already heard much about insulin, and we will hear more on Sunday. I am sure that Dr. Houssay and Dr. Randle will deal with the growth hormone tomorrow. Therefore, let us begin with the estrogens, which are summarized briefly in Table I. I

TABLE I

Estrogens

Physiological Effects	*Enzyme Responses in Vivo*	*In Vitro Effects*
Uterine growth (Protein synthesis)	Increased glucuronidase in target organs, not in liver, kidney, or spleen	
Mammary gland growth	Increased citrate oxidation	Activates isocitric dehydrogenase of placenta
	Increased formate incorporation	2, or 4 hydroxy estradiol activates formate incorporation in uterine strips
	Peroxidase appears in uterus.	

cally discrete material is reduced. Do you find this is associated with any great change in the polydispersion?

DR. SETTEN: We didn't test the dispersion of such samples but the weight-averaged molecular weight in animals fasted for a long period of time and presumably histologically depleted of stainable glycogen granules was, if anything, a little higher, certainly not lower than the corresponding molecular weight of animals well nourished and full of stainable glycogen granules.

estradiol has little or no effect. Now these are interesting findings, but some aspects of the experiments dampen my enthusiasm. The first is that in an earlier paper Villee presented data showing that estradiol stimulated lactate production from pyruvate just as much as it stimulated keto-glutarate production. I think it would have been interesting for them to follow up the lactic dehydrogenase as well as the isocitric dehydrogenase. But, no mention has been made of that phase of the problem. An unexplained incongruity is the fact that when lactate is added as a substrate, estradiol enhances the accumulation of lactate in the medium, though I don't know where it comes from. In balance studies with dialyzed enzyme preparations, in which there is no endogenous keto-glutarate production, and under anaerobic conditions where there is, by definition no oxygen uptake, more keto-glutarate is produced from iso-citric acid than one would predict from the amount of hydrogen acceptor present. They used DPN and methylene blue as hydrogen acceptors. These should give stoichiometrically, a mole of keto-glutarate for each mole of the hydrogen acceptor reduced; yet more keto-glutarate is produced than the amount of hydrogen acceptor added. I also notice that in their other balance studies there is more keto-glutarate production than there is citrate or iso-citrate disappearing. This leads one to question the analytical procedures. I think the spectrophotometric studies of the dehydrogenase where DPN reduction was measured, are quite convincing. The concentration of estrogen required for the *in vitro* effect should be pointed out; Dr. Astwood and I made a rapid calculation this afternoon and decided that 4×10^{-6} was at least two orders of magnitude higher than one would expect to find under physiologic conditions.

Finally I think it is difficult to rationalize this effect of estradiol with some observations which Dr. Roberts and his wife published some years ago on the uterus of the rat. Within four hours of estrogen treatment, they found a greatly enhanced aerobic and anaerobic glycolysis, with no effect on oxygen uptake. The latter increased only after about 20 hours when the tissue was very actively proliferating. Incidentally I believe that Drs. Roberts and Szego interpreted their results to indicate that estrogens had an effect on permeability of the uterine wall.

have listed the physiologic effects, some *in vivo* enzymatic responses to administered estrogen, and some *in vitro* effects of estradiol or derivatives thereof. In the case of glucuronidase, there is a tremendous increase in the enzyme following estrogen treatment. There is no agreement about the significance of this finding. Recently Dr. Stotz and coworkers at Rochester have demonstrated a tremendous increase in peroxidase content of the uterus following estrogen treatment. One still can't say what this means in terms of the function of the hormone. Perhaps the most specific response reported is that observed by Villee and his coworkers. In 1952 Hagerman and Villee (*Arch. Biochem. & Biophys. 40*:481, 1952)—found that estradiol would increase the oxygen uptake of human endometrium when it was added *in vitro*. These workers have recently clarified the site at which this effect is exerted. They used 4 $(10)^{-6}$ molar estradiol in most of their experiments. Estrone is equally effective. Progesterone, diethylstilbesterol, and estriol were also effective if they were present in a concentration ten fold that of estradiol. Their original effect was one of stimulation of oxygen uptake, with pyruvate as a substrate. On examination they found that this effect seemed to be limited to an oxidation in the Krebs cycle, which they traced to the enzyme isocitric dehydrogenase. They found that isocitrate disappears more rapidly in the presence of estradiol than in the control experiments. This effect is specific for a DPN-linked isocitric dehydrogenase, which appears in the supernatant fraction of a placental homogenate.

We now switch from endometrium to human placenta, which is the tissue used in their more recent work. With triphosphopyridine nucleotide rather than DPN, the rate of citrate oxidation is greater than it is with DPN, but no estradiol effect can be observed. If the substrate concentration is increased, to I believe five micromoles per flask instead of one, then again there is no stimulation by estrogen. The stimulation of the DPN-linked enzyme, at low substrate concentration, shows specificity, since non-target organs like liver and kidney do not respond to estradiol. The pH optimum for the effect of estradiol is at 7.2, a pH at which the isocitric dehydrogenase has very little activity in the absence of estradiol. At pH 8.2 where they had been measuring it previously, there is much less stimulatory effect, and near pH 9.0

saline buffer for 40 minutes. It was established that the phosphorylase reaction was the rate-limiting one, in the conversion of glycogen to glucose by the liver slice. Following such incubation, more phosphorylase activity could be demonstrated in the slices treated with epinephrine than in the controls. In rat diaphragm, phosphorylase-a is largely converted to phosphorylase-b (active only with adenylic acid) during a 20 minute incubation period. The addition of epinephrine caused a rapid reconversion of the b-form to phosphorylase-a.

In more recent work Sutherland has found that purified liver phosphorylase contains phosphate. An enzyme which inactivates the phosphorylase splits the phosphate from the enzyme protein. When liver slices are incubated with epinephrine, radioactive phosphate is rapidly incorporated into the phosphorylase which can now be isolated in the enzymatically active form.

There can be no doubt that these *in vitro* effects of minute amounts of epinephrine are of tremendous importance. It seems likely, however, that the conversion of inactive phosphorylase to an active form cannot be the sole regulating influence of epinephrine on glycogen breakdown. We must recall that the amount of enzyme does not significantly influence the equilibrium point of a reaction. As Dr. Stetten has pointed out it is hard to see how making a fraction of this enzyme more active through the aegis of epinephrine could influence the direction that phosphorylase takes If we assume that the liver glycogen is available to phosphorylase, and that it has been synthesized by this enzyme, activating a portion of the phosphorylase should not, it would seem, cause a sudden change in the direction of the phosphorylase reaction.

The amount of glycogen synthesized is dependent on the concentrations of inorganic phosphate and of glucose-1-phosphate. When an equilibrium is reached in this system, the sudden activation of an enzyme which catalyzes the attainment of that equilibrium should not cause the glycogen to be converted to glucose-1-phosphate.

How, then, can we explain the fact that epinephrine enhances the formation of hexose monophosphate in muscle and increases the formation of glucose in liver? Perhaps this hormone too influ-

The other *in vitro* observation which is of interest is that reported by Dr. Gerald Mueller (*Nature, 176*:127, 1955). He finds that giving estrogen to a female rat a few hours before it is sacrificed greatly enhances formate incorporation into strips of the uterus *in vitro*. When any of the known estrogens are added directly to the uterine strips *in vitro*, there is no enhancement of formate incorporation. However, when synthetic 2-hydroxy and 4-hydroxy estradiol are added to the uterine strip, *in vitro* formate incorporation is greatly enhanced. It will be interesting to see if these compounds turn out to be intermediates in estrogen metabolism.

EPINEPHRINE

The physiological effects of epinephrine are perhaps more varied than those of any other hormone. Because of its profound effect on blood sugar, epinephrine has been used in many *in vitro* studies of carbohydrate metabolism. Effects are relatively easy to demonstrate with perfused organs, diaphragm pieces, or even with liver slices. No effects related to its *in vivo* activity have been observed with cell-free preparations of any tissue (Table II).

Sutherland and Cori discovered a dramatic effect of minute amounts of epinephrine *in vitro*. Glucose liberation was found to be greatly enhanced by the addition of as little as 1 gamma of epinephrine to a liver slice incubated in 1.2 ml. of phosphate-

TABLE II

EPINEPHRINE

Physiological Effects	*In Vitro Effects*	*Enzyme Responses*
Glycogenolysis Plasma phosphate decreases H.M.P. accumulates	Glucose liberated from liver slices	Restores phosphorylase activity in aged liver slices. In diaphragm stimulates phosphorylase b→a
O_2 utilization increased	No effect on diaphragm	
Increased blood pressure	Enhances transport of Na^+ across frog skin	

enzyme level (Table III) but one which is capable of being studied by the biochemist is the efficiency with which energy liberated by oxidative processes is trapped in a utilizable form by surviving tissue preparations. By comparing tissues from euthyroid animals with those from hyperthyroid or hypothyroid animals, it has been found that excessive amounts of thyroid hormone decrease the efficiency of energy trapping. There is some evidence that even the small amounts of thyroid hormone in the normal animal may

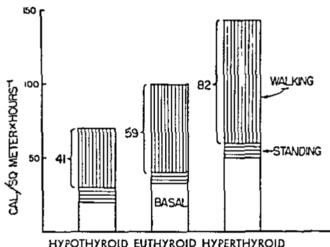


FIGURE 1.

decrease the efficiency of the energy-transfer mechanisms. In the intact human subject there is a great deal of evidence that the hyperthyroid person is a less efficient metabolic machine than is the normal. There is some evidence to indicate that the hypothyroid subject is a more efficient metabolic machine. The work of Briard, McClintock and Baldridge (*Arch. Int. Med*, *56*:30, 1935) is summarized in Fig. 1. It is substantiated by that of Simonson (*Arch exper. Pathol & Pharmacol.*, *120*:259, 1927) and of Hilde Bruch (*Jahrb. Kinderh.*, *121*:7, 1928) In each of these studies it was found that fewer calories were expended by hypothyroid subjects to accomplish a given amount of work than was the case with normal or hyperthyroid subjects. There are many factors involved in measuring over-all efficiency of work performance. Because these factors are not readily subject to experimental control, con-

ences cell permeability--in this case to inorganic phosphate. Epinephrine is known to effect a decrease in plasma inorganic phosphate. If this hormone acts to permit phosphate to diffuse into liver and muscle cells, the increased inorganic phosphate would favor glycogen breakdown, with resultant glucose production in liver, and hexose monophosphate and lactate accumulation in muscle. Perhaps, Dr. Best would like to discuss this too, for a similar phenomenon occurs in the case of insulin treated animals.

Glucagon which also was discussed this morning, is a polypeptide hormone which may be produced by the pancreatic alpha cells. It acts like epinephrine in liver, but appears to have no direct effect on muscle.

THE THYROID HORMONE

The thyroid hormone affects a tremendous variety of biological processes, including work, metamorphosis, and brain function. Very few of these processes are amenable to examination at the

TABLE III

THYROID HORMONE

Physiological Effect	*Enzyme Response In Vivo*	*Enzyme Response in Vitro*
Enhanced O_2 consumption	Cytochrome c, succinic dehydrogenase, glutamic and Krebs cycle oxidation enhanced in liver, muscle	10^{-7}M T_3 acetic increases O_2 use by kidney and liver slices 10^{-6}M T_3, T_3 propionic increase O_2 use by kidney and liver mitochondria.
Decreased work efficiency.	Decreased P/O of isolated mitochondria.	T_4, T_3, and analogs uncouple oxidative phosphorylation.
Stimulates growth and protein synthesis. Excess inhibits growth.	Enhanced uptake of labeled compounds, depressed by excessive doses of hormone.	

T_4 = thyroxine.
T_3 = triiodothyronine.
T_3 Acetic = triiodothyroacetic acid.
T_3 Propionic = triiodothyropropionic acid.

oxidized to succinate, by a system which involves none of the electron transport enzymes about DPN, can be measured with an efficiency of 0.8. Exactly the same efficiency of phosphate uptake is obtained when this step is measured with mitochondria from hyperthyroid rats, indicating the hormone has no effect on this phosphorylation step.

Another system permits measurement of the single phosphorylation which occurs when cytochrome C is oxidized by oxygen. Added cytochrome C is reduced with epinephrine. The reduced cytochrome C is then oxidized through the cytochrome oxidase system of the mitochondria. This system yields a P/O ratio of about 0.7 with normal mitochondria and about 0.5 with mitochondria from hyperthyroid rats. It appears that neither of these

TABLE V

←———————— 2.7 —— (1.8) ————————→

Hydroxybutyrate	DPNH	FlH_2	Cyt b^{++}	Cyt c^{++}	Cyt a^{++}	H_2O
Acetoacetate	DPN	Fl	b^{+++}	c^{+++}	a^{+++}	O

←— 1.3 —— (0.7) —→

single-step phosphorylations is appreciably effected by the hyperthyroid state. One may assume that one of the other two phosphorylations which cannot be studied as single-step reactions is probably more strongly affected. This is borne out by experiments with beta-hydroxybutyrate as a substrate (Table V). Under such conditions there is no phosphate-fixing step prior to DPN and the theoretical P/O in 3. The over-all phosphorylation efficiency is decreased about one-third in the mitochondria from hyperthyroid animals. When ferricyanide is used as an electron acceptor the first two phosphorylation sites are involved and one can measure the uptake of 1.3 moles of phosphate per mole of substrate oxidized. With mitochondria from hyperthyroid rats this is decreased to 0.7. This again indicates that thyroid hormone affects one or both of the phosphorylations occurring between DPNH and cytochrome B.

This decreased efficiency of coupling phosphorylation with oxidation might easily account for the higher basal metabolic rate and decreased work efficiency of hyperthyroid animals. The question to be answered is whether the anabolic and beneficial effects

clusions must be drawn with care. The nearest counterpart one may study *in vitro* is the measurement of phosphate uptake by mitochondria from experimental animals in various states of thyroid activity. The basis for this statement is as follows. In animals, as well as in most aerobic microorganisms, the greater part of the energy required for cell growth and for work of all sorts is liberated [illegible] of "h [illegible] phosphate uptake and conversion to organic phosphate is so closely geared to the process of cellular respiration, that respiration rate is closely parallel to rate of work performance. (Lardy: *Proc. Third International Congress of Biochemistry*, p. 287-294, 1955.)

TABLE IV

←——— 3 6 —— (2.7) ———→												
αKetoglutarate		DPNH		FlH_2		Cyt b^{++}		Cyt c^{++}		Cyt a^{++}		H_2O
⇅	→	⇅	→	⇅	→	⇅	→	⇅	→	⇅	→	↑
Succinate		DPN		Fl		b^{+++}		c^{+++}		a^{+++}		O
← 0 8 (0 8) →										← 0.7 (0.5) →		

Although the actual mechanism of phosphate uptake is not known, something can be said about the enzymes involved in the process. The pathway of oxidation of a substrate like alpha-ketoglutarate is outlined in Table IV. For each mole-equivalent of keto-glutarate oxidized, four atom equivalents of inorganic phosphate are converted to a bound form capable of reacting with adenosine diphosphate to yield adenosine triphosphate. One mole of phosphate is fixed at the level of the reaction between DPN and alpha keto-glutarate and another between pyridine nucleotide and flavin. A third is fixed somewhere in the vicinity of cytochrome B and the fourth is somewhere between cytochrome C and oxygen. One can measure *in vitro* the uptake of, on the average, 3.6 moles of phosphate for each atom of oxygen consumed by liver or kidney mitochondria. In mildly hyperthyroid rats, the efficiency of phosphate uptake has been decreased to 2.7 moles of phosphate per atom of oxygen consumed (figure in parentheses, Table IV. The data are from Maley and Lardy: *J. Biol. Chem.*, *215*:377, 1955).

The single phosphorylation occurring when the substrate is

to normal by therapy. There are a number of *in vitro* effects reported for adrenal cortical hormones, but it seems to me that none of those are very specific, because the concentrations required to obtain significant effects are approximately 10^{-3} molar and I am sure that is much higher than we could reasonably expect under any conditions in nature. There are a number of bits of evidence to indicate that the effect of the adrenal cortical hormone is exerted between amino acids and proteins. In the extra hepatic tissues the protein-to-amino acid conversion is stimulated by the adrenal cortical hormones, and free amino acids are being formed at greater than a normal rate. Amino acid excretion is greater than normal in the patient treated with ACTH or cortisone. And more of other nitrogenous compounds are being excreted in the urine while more carbohydrate is being synthesized from the carbon chains of these materials. If an adrenal cortical hormone were uncoupling the reaction which regulates balance between protein and amino acids we might expect such results.

There is one possibility that I don't think has been mentioned in the literature, and which I would like to bring up now. That is the possibility that an adrenal cortical hormone affects the oxidation of a single essential amino acid. Let us, as an example, say that cortical hormone enhances the oxidation of amino acid "x" to the nitrogen-free residue, and thereby makes it unavailable for protein synthesis. If that were the case then, no protein synthesis could occur, because when a new protein molecule was being synthesized there would be no nut to put on the bolt when the molecule gets to the "x" stage. The machine would have to be discarded. If the hormone stimulated the oxidation of a single specific essential amino acid, it might effect a decreased protein synthesis and indirectly an enhanced glucose synthesis from the fragments of the other amino acids as well. Experiments to test this possibility should be carried out

DISCUSSION

DR. GRIFFITH: We are assuming that there is no question about the fact of hormonal regulation of enzymatic activity. This, of course, seems reasonable because we associate metabolic reactions with enzyme catalysis. If there is an additional effect of hormones

of the hormone could be exerted through uncoupling of phosphorylation from oxidation.

We have discussed elsewhere (Symposium on *The Biology of Phosphorus*, Michigan State College Press, p. 131, 1952) the conditions required to obtain a beneficial effect from *specific* uncoupling of a phosphorylation which is limiting the rate of a sequence of reactions. This beneficial effect might or might not be exerted through its effect on the amount of "high-energy" phosphate delivered to the cell. It might be exerted by enhancing the rate of oxidations which give rise to products which are used catalytically in other systems. Uncoupling could conceivably enhance growth and protein deposition by providing a greater concentration of nucleotides in the diphosphate form which is used for nucleic acid syntheses.

ADRENAL CORTEX

I should like to close with a few words about the adrenal cortical hormones without encroaching on Dr. Astwood's territory. One of the most outstanding effects of adrenal steroids is the increased nitrogen excretion resulting from protein breakdown (Table VI). Some enzyme responses which have been observed in adrenalectomized animals are the decreased proline oxidation by kidney tissue and the decreased D-amino oxidation in liver. Administration of cortisone brings both back up to the normal level. In liver there is no difference in proline oxidation between the normal and the adrenalectomized animal. These are experiments of Umbreit at the Merck Institute. These are not all-or-none changes, since the values drop about 50%, and are brought back

TABLE VI

ADRENAL CORTICAL HORMONES

Physiological Effects	*Enzyme Response in Adrex Rats*	*In Vitro Effects*
Enhanced N excretion	Cortisone enhances proline oxidation by kidney to normal level	Non-specific inhibition by high concentration.
Glucogenesis from protein	No proline oxidation change in liver D-Amino acid oxidation brought back by cortisone.	

tion. For example, in Engel's experiments where amino acids were administered together with corticosteroids, the catabolic effect of the corticosteroids was prevented. There was no evidence for corticosteroid stimulation of amino acid oxidation under those circumstances. In line with Dr. Griffith's remarks, I would like to suggest that the influence of the corticosteroids on protein metabolism may be secondary to effect on cellular permeability. Some experiments we carried out suggested, as a matter of fact, that the corticosteroids may act to mobilize tissue protein. Once tissue protein is mobilized, its utilization may depend upon the protein demands of the organism at that particular time. This protein may then be used either for catabolic purposes, if there is a requirement for energy, or it may be used for anabolic purposes, for example, in the instance where the liver has been damaged and where the restoration of liver protein is a requirement. In this regard, it has been demonstrated in our laboratory that cortisone or ACTH enhances the regeneration of liver tissue without resulting in over-all increase in protein metabolism (*cf.* Roberts, S.: *J. Biol. Chem., 200:*77, 1953).

DR. STADIE: I would like to ask a question of Dr. Lardy, hoping that he can clarify some confusion in my mind. He spoke of the necessity of the action of uncoupling agents as a part of the chain of physiological homeostasis in tissues, and the possible role of thyroxin or its analogues in such a function. I am puzzled by the paper of Krebs* in which he reported experiments using P^{32} with mitochondrial preparations together with suitable substrates. These experiments were done in the absence of phosphate acceptors and he reported an undiminished oxygen uptake at a P:O ratio of approximately three to four. In other words, oxidation *goes on at a normal rate in the absence of uncoupling* agents or phosphate acceptors. I believe these experiments were confirmed by those of Lee† published in the *Journal of Biological Chemistry.* I have difficulty in reconciling these two experimental results

* Krebs, H A, Ruffo, A., Johnson, M, Eggleston, L V, and Hems, R.: *Biochem. J, 54* 107, 1953.

† Lee, K H., and Eiler, J J: *J Biol Chem, 203* 705, 719, 1953

on metabolism, it is reasonable to think of this effect as related to the enzymatically catalyzed reactions which predominate. I have been impressed with two recurring thoughts in connection with the remarks so far in these two sessions. One concept of the possible hormonal regulation of metabolic processes involves the permeability of membranes. If there is a change in the rate at which metabolites pass through membranes, this effect may be nothing more than a concentration and mass law effect. It may well be that this is one of the types of activity of the hormones. The other very different concept involves a direct effect, presumably upon the enzyme system itself. In order to bring these two types of activity into sharper focus it is suggested that each speaker emphasize in his discussions which of these two effects may be primarily involved in the experimental findings which are being presented. Dr. Lardy's very interesting and provocative remarks are before you for discussion.

DR. BEST: Dr. Lardy mentioned changes in inorganic phosphate with insulin. It was Wigglesworth and his colleagues who first saw this effect in 1922. Shortly, thereafter, several groups in this country Blatherwick; Perlywhig; Harrop and others studied the fall in inorganic phosphate in blood after insulin, but the interrelationship of epinephrine and insulin in this effect has been confused. Dr. Levine and his colleagues have contributed to this subject recently and he may wish to bring us up to date.

DR. RANDLE: Groen and Willebrands state that insulin does not increase the uptake of inorganic phosphate by the isolated rat diaphragm *in vitro*, though insulin did increase the uptake of potassium (Groen, J. and Willebrands, A. F.: *Science, 111*.30, 1950).

DR. ROBERTS: I should like to return to Dr. Lardy's remark on the effects of corticosteroids on protein metabolism. He suggested that the basic mechanism might involve stimulation of amino acid oxidation in the liver. I think this is somewhat unlikely in view of the fact that protein synthesis in the liver appears to be enhanced, in some instances at least, under corticosteroid stimula-

as such in tissues of animals pretreated with adrenocortical hormones as compared with normal or adrenalectomized animals. What has been demonstrated, and is the basis for my suggestion concerning the possible role of the adrenocortical hormones in labilizing tissue protein, is that various tissues removed from adrenalectomized, normal, or corticosteroid-treated rats show a differential rate of protein released to a serum medium *in vitro*. Thus, hepatic and splenic tissue from animals injected with corticosteroids exhibit a greater release of protein under these circumstances than similar tissues from normal or adrenalectomized rats. These results, along with others obtained in *in vivo* experiments, suggest that there may be an influence of these hormones on mobilization of tissue protein. It's tempting to theorize that there might be but a single action of the corticosteroids on protein metabolism. It would appear that this action is permissive, as Ingle has frequently pointed out, in that the disposition of mobilized protein appears to depend upon the physiological state of the organism.

DR SAMUELS: I think that I might add a few items that have a bearing on this problem, and I think support Dr. Roberts' views. If we force-feed adrenalectomized animals a diet deficient in one amino acid (I will speak particularly of isoleucine deficiency because this is the one we have studied most), such an animal dies on the second or third day without any obvious signs, other than it becomes weak an hour or so before death If we include the missing amino acid, the animal gets along very well. If we inject cortisol, the animal then also gets along well without the addition of the amino acid, and eventually develops symptoms of deficiency similar to those of the intact animal on the deficient diet. So that it would appear that this animal without the adrenal develops a fatal situation rather quickly. Now if we study the transfer of protein, we find that the skeletal muscle and the skin lose protein during this period of amino acid deficiency and injection of cortisol. The liver, however, maintains its concentration quite well; the heart maintains its concentration, the brain, which usually within a few days will have a low level without the injection of cortisol, with the injection of cortisol has the same nitrogen at the end of 14 days as the original animal. So that it looks as

which appear to be anti-thetical—Krebs' and Lee's work on the one hand, and your results on the other.

DR. LARDY: Krebs was not measuring net fixation of phosphate. He measured the incorporation of tracer doses of P^{32} into nucleotides by the mitochrondrial preparation. The rate of respiration was presumably much lower than we would expect in a system where there is a phosphate acceptor present. He calculated the P:O ratio from the rate of P^{32} incorporation. This has given results which in general are consistent with those that are done by the analytical procedure. With keto-glutarate he gets a value above four, approximately five; and with succinate, I believe his value is more like three than two. Most people are inclined to believe that there are some sources of error, and these have been pointed out in the literature. One source of error is the possibility of exchange reactions which are not connected with the oxidative phosphorylase mechanisms. These have been described in the literature by Boyer at Minnesota and by Mildred Cohn at St. Louis. I don't think there is any controversy between Krebs and ourselves on the question of the regulating influence of phosphate acceptors. His system is running at the "basal" rate; ours is running wide open, so to speak. Krebs' machine is "stopped down." His respiration rates must perforce be less than they would be if phosphate acceptor were present.

In coming back to Dr. Roberts question, I know that it is quite true that fed amino acids or proteins seems to be used perfectly well. There is a possibility that during the period of feeding and amino acid absorption, you have an abundance of all amino acids and therefor protein can be synthesized at the time. But it could not be resynthesized once the concentrations of the rapidly oxidized amino acid became extremely low. I hold no brief for that mechanism. I should like to think that cortical hormone is more specifically involved in the energy-utilizing step by which amino acids are converted to protein We really know very little about the energy coupling mechanisms involved in the conversion of amino acids to proteins.

DR. ROBERTS: If I may answer that—I don't know of any *in vitro* experiments which have shown a difference in protein catabolism

siderable increases in ribonucleic acid concentrations in the liver, and on the basis of some preliminary and incomplete data, there seems to be a decrease in ribonuclease activity. The meaning of the latter I don't know. It would seem that these effects may perhaps be associated with a rather different reaction going on in the liver than in the periphery, and does suggest that one needs to look for different effects, in skeletal muscle for example, and in other organs, much as we have to with reference to insulin effects in the liver and extra hepatic sites.

Dr. Gross: I would like to return to the thyroid and ask two questions. First, Dr. Lardy's scheme depends upon the specific points of action, for thyroid hormone to exert its activity.

Dr. Lardy: You mean the hypothesis, as to how it might work?

Dr. Gross: Yes, as I recall in reading Hoch and Lippman's paper, they felt that the uncoupling effect was an all-or-none phenomenon. I wonder if you could discuss this? Secondly, I wonder if you would describe any results that you obtain in hypothyroid animals compared with the consistently hyperthyroid which you used in relation to this phenomenon?

Dr. Lardy: Well, with respect to the first question, Hoch and Lippman under some circumstances did get a zero phosphorylating efficiency in the mitochondria of their animals, but as you will recall they received tremendous doses of thyroxin by injection, I believe as much as 16 milligrams per rat per day. Undoubtedly these animals would not have survived very long if they hadn't been sacrificed for the experiment. So I think we can not draw conclusions about the specificity of the uncoupling from animals which are in that state of thyroid activity. We have done many, many experiments with hypothyroid rats, and in general can find no difference in the P:O ratios of the mitochondria. But one way that we have been able to get at the problem is to measure the rate of respiration of normal, hypo, and hyperthyroid rat mitochondria in the absence of the phosphate acceptor. In that case, the hypo-

though there is this matter of transfer. Now if we study the amino acid content of plasma, the adrenalectomized animal on a deficient diet shows very high levels of the essential amino acids other than the deficient one. Isoleucine is absent unless cortisol is injected.

DR. LARDY: May I ask a question please? Does compound F enhance nitrogen retention in the absence of isoleucine?

DR. SAMUELS: Not total nitrogen retention, but the nitrogens of the liver, brain and heart are maintained, the animal gets along satisfactorily, and the amino acids do not accumulate in plasma.

DR. LARDY: How do you interpret these experiments?

DR. SAMUELS: Well, I interpret them as indicating that cortisol mobilizes amino acids from the skeletal muscles and other less vital sources, making them available for other tissues. There is a differential priority in the utilization of the circulating amino acids. Certain essential tissues pick up the amino acids which have been released from the other tissues, and synthesis goes on to the point where the acid finally becomes a limiting factor in a number of structures. Without cortisol, the missing amino acid becomes almost immediately a limiting factor, if it is removed from the amino acid milieu that the animals receive. The accumulation of the other amino acids also may indicate some interference with the deaminative or transaminative reactions.

DR. ZELDIS: In line with Dr. Samuels' remarks, we have some pertinent data. In some incomplete experiments in which we are studying the incorporation of N-15 labelled amino acids into the livers of intact animals injured with subcutaneous turpentine injections, we note an effect which we think is in part, at least, a corticoid-like effect. We find that not only is the rate of lysine incorporation into the liver proteins increased in injured animals, but that there is actually an increase in total protein concentration of the liver. This does suggest that there is a mobilization from the periphery to the liver. Associated with this are quite con-

Dr. Levine: The drop in P:O ratio may be the effect of an overdose of thyroid rather than the primary effect of the hormonal action in the normal organism. P:O fall is somehow incompatible with group enhancement, metamorphosis etc. That's why I brought up this term "efficiency" which is difficult to measure. The data you showed could not be said to have measured "efficiency."

Dr. Lardy: In *in vitro* studies, or even *in vivo* studies, with *large doses* of thyroid hormone, I think we are measuring an uncoupling effect produced by halogenated phenol. Almost any halogenated phenol would give you this effect. I think that there is a possibility however, that the beneficial effect of physiological doses of the thyroid hormone are exerted by uncoupling, but at a specific site. It's entirely possible that the thyroid hormone is converted into something much more active, and as you know, Dr. Pitt-Rivers believes that one such agent might be triiodothyroacetic acid, which shows *in vitro* effects at much lower concentrations than one can obtain with thyroxin or triiodothyronine. She and Dr. Thibault actually have responses at 10^{-7} molar. These are increases in rate of oxidation of kidney slices which might reflect an uncoupling. Dinitophenol would certainly give the same effect but at a higher concentration, I believe. We havn't had much triiodothyroacetic acid available to us, but we have synthesized an analogue of that compound, namely triiodothyropropionic acid. This compound has a fair degree of activity in an intact rat, both in the goiter prevention assay and in the basal metabolic rate stimulation assay. It gives a much better *in vitro* response, with mitochondria than do thyroxin or triiodothyronine.

Dr. Szego: I am sure, Dr. Lardy that you have been asked this question before, but do you visualize a similar mechanism as operative in differentiation, as for example in metamorphosis?

Dr. Lardy: I have no idea of how the process of metamorphosis is brought about in terms of energy transfer mechanisms. If the hormone effects are exerted at the nucleotide level, the process of differentiation could easily be influenced. Simply increasing the

thyroid rats yield mitochondria which have a lower rate of respiration than a normal animal, and the normal animal in turn has mitochondria which have lower rates of respiration than the hyperthyroid rats. This indicates that the coupling mechanism which is controlling respiration when there is no work imposed on the mitochondria, seems to be a little bit better in the hypothyroid rats. These data were published in the *Journal of Biological Chemistry* last July, and those are the only data that we have that would indicate greater efficiency in the hypothyroid animal.

DR. LEVINE: *Dr. Lardy,* I refer to work from Lippman's laboratory in which hypothyroid rats with and without substitution therapy were used. Liver slices were removed and amino acid incorporation was measured. The hypothyroid animal showed a lower amino acid incorporation capacity than did the normal, and the incorporation capacity was restored by prior administration of thyroxin (8). Would you call that a decreased efficiency? It would seem that this was probably a measure of synthetic capacity of the tissue and the utilization of energy for building up a more complex molecule for amino acids. It does seem that the hypothyroid was less efficient than the normal or the treated hypothyroid animal.

DR. LARDY: I don't think we can say whether the hypothyroid was more or less efficient on an energy basis unless we measure the energy cost of synthesizing the protein. And that obviously can't be done as yet.

DR. LEVINE: The oxygen consumption was less in the hypothyroid.

DR. LARDY: Oh, yes.

DR. LEVINE: And the amino acid incorporation was less.

DR. LARDY: The fact that thyroid hormone stimulates amino acid incorporation or any of a variety of other synthetic reactions cannot be doubted at all.

• DUTOIT, C In *Phosphorus Metabolism*, Baltimore, John Hopkins Press, 2 597, 1952.

would inhibit unspecifically, and most of the reactions have been inhibitory in nature. Therefore, I have tended to reserve my judgment as to their real effect on the enzymic system itself. There are a few experiments, however, in which this doesn't seem to apply, but I haven't studied these carefully.

DR. LARDY: I was thinking more of experiments in which androgen was administered to the intact animal, and then subsequently tissues were excised and the concentrations of certain enzymes were determined. I think that what we measure is really an adaptive response to some other physiological change. For example, if the amino acid concentration changes, the amount of the enzymes that handle the amino acid will alter in response to the increased substrate concentration.

DR. STADIE: Dr. Lardy, would you clarify our understanding of the work you have reported on the function of the uncoupling of oxidative phosphorylation when thyroxin is included in the equilibrating mixture with mitochondria? Lehninger, if I recall correctly, reported experiments in which he apparently demonstrated oxidative activity in acetone powder prepared from mitochondria. These preparations, presumably particulate free, *showed about the same phosphorylative activity as did intact* mitochondria. The addition of dinitrophenol resulted in uncoupling, but no uncoupling was reported when thyroxin or its analogues were used.

DR. LARDY: Lehninger's experiments were done, not with acetone powders of mitochondria, but with submicroscopic particles which are obtained by dissociating the mitochondria with a detergent, digitonin. This technique has been used by Colowick and by Raw in South America. Lehninger has obtained very good phosphorylation efficiency in these particles. It is assumed by workers in this area that this represents a further reduction in size of the mitochondrium, but that the particle is still more or less intact, since it has all the co-factors and enzymes necessary for the oxidation and phosphorylation of substrates. He obtained no uncoupling with thyroxin, but that is not a unique situation. A good many

concentration of nucleotide diphosphate could conceivably enhance nucleic acid synthesis. During the last several years, more and more minor components, in a quantitative sense, have come to be disclosed as being absolutely essential for some metabolic processes. For example, the work of Kennedy, showing that cytidene diphosphocholine and cytidene diphosphoethanolamine are essential compounds in the synthesis of phospholipid. The work of Leloir and his group really opened up this whole area, with their discoveries of uridene diphosphoglucose and uridene diphosphogalactose, and uridine diphosphoacetyl glucosamine. These compounds are turning up as the essential intermediates in syntheses. Perhaps some of the hormones are exerting their effect at one of these "trigger" nucleotides, because extremely small amounts of most of the hormones are effective in bringing about rather major transformations. As Dr Houssay pointed out, hormones are not essential for the basic life processes, but they seem to be necessary for altering the rates at which these processes are carried out. So, controlling the direction of these nucleotide syntheses would be one way by which very minute amounts of hormones could exert their effects.

DR. KINSELL: Do you have any information regarding the action of any of the anabolic steroids, Dr. Lardy?

DR. LARDY: A lot of experiments have been done with testosterone and there are very profound effects on certain enzymes, yet the people who are working in this area are not yet ready to state that these enzyme changes are responsible for the expression of activity of the hormone. I have done no experiments with testosterone so I have no first hand information to offer. Dr. Samuels has worked in that area, and would perhaps have more information to offer.

DR. SAMUELS: I have not worked specifically with these enzymic reactions, but several workers have reported the effect of testosterone. In many cases, of course, the concentrations are high, and often exceed the solubility of the steroid in the medium. This means that the steroid can very readily form surface films that

DR. LARDY: We do not yet know very much about the enzymatic processes involved in protein synthesis. Several papers indicate that amino acids are incorporated into protein by forming the adenyl derivative. All of these are based on an extension of the experiments of Berg, on the mechanism of acetate activation. Berg found that the acetic acid molecule is activated, that is, it is permitted to carry out various reactions, by reacting with adenosine triphosphate to split off pyrophosphate and leave the acetyl hooked to the terminal phosphate of the adenylic acid portion which is left. This reaction now can react with co-enzyme A to synthesize acetyl co-enzyme A, and leave adenylic acid as the end product. The acetyl co-enzyme A can be used for a wide variety of acetylation reactions, for fatty acid synthesis, and so on. Hoagland, Borsook and others have postulated that amino acids are converted to protein by forming analogous compounds. Factors affecting the nucleotides might in this manner affect protein synthesis, and maybe those minor nucleotide molecules in a quantitative sense, are the things that are really triggering many of these mechanisms.

DR. ASTWOOD: Do you think that if you could stimulate protein synthesis, that that might be followed by cellular growth?

DR. LARDY: I don't know whether protein synthesis is a sufficient stimulus to cell division.

DR RANDLE: Dr. Lardy can you visualize, on this basis, any mechanism whereby fat catabolism could stimulate protein synthesis? Greenbaum reported that rats would grow in response to growth hormone until the available fat reserves had been utilized. When this had occurred resistance to the growth promoting action of the hormone was seen (Greenbaum, A. L.· *Biochem. J., 54*:400, 1953).

DR LARDY There is a possibility of course that fat has a specific function other than supplying energy. We do know that in the case of fatty acid activation we can get exchange reactions on

people have failed to obtain *in vitro* effects with thyroxin. These particles contain a great deal of digitonin and possibly it prevents the large thyroxine molecule from gaining access. Also, it is possible that once the mitochondrion has been disrupted there is no longer a conversion of the thyroxin to a more active form. I might mention that a couple of years ago, after Drs. Albright and Larson demonstrated that kidney slices can effect the conversion of thyroxin to triiodothyronine, we began work in collaboration with them on the enzymatic aspects of this problem. We are now engaged in isolating a substance which is produced by mitochondria and by water soluble extracts of mitochondria. This compound has biological activity, and it may be that thyroxine is converted to this material before it becomes active at the enzyme level.

DR. GROSS: If I can quote our discussion of yesterday when we talked about this privately, wasn't it so that you could not get this compound in the intact cell?

DR. LARDY: In the kidney slice there was no appreciable accumulation of this compound. In the cell free preparation as much as 60% of the added thyroxin can be converted to the unkown compound in an hour or two. In the intact slice much of the thyroxin is converted to triiodothyronine.

DR. GROSS: Do you still feel that this may be an important metabolite then in the sequence of thyroid hormone action?

DR. LARDY: Well, I think there may be some further metabolite which is important, but whether this compound is, has yet to be established.

DR. ASTWOOD: I would like to ask a question, Dr. Lardy. Many of the hormones when they exert their specific effects bring about profound alterations in tissues, including growth. It is difficult for me to envision any kind of specific action on a step in intermediary energy transformation that, if affected specifically by a hormone, would lead to cellular growth and proliferation.

are bound to the mitochondria, and they are bound very tenaciously. After the first wash, only a trace of radioactive thyroxin or triiodothyronine can be removed from the mitochondria in a salt solution, which will displace anything that is bound ionically. Hoch and Lippman have since published in one of their papers similar results. In our case the total amount of material bound was far in excess of what one would ever expect to encounter under physiological conditions. I think the binding must be physiologically nonspecific.

DR. GROSS: In this connection there are some data on the distribution of I^{131} on cellular particulates in the liver after radioactive thyroxin administration (a, b). As I recall, with the labelled material there is somewhat less in the mitochondria than in other fractions, and in the case of Riggs' (c) data on the protein bound iodine of these fractions in untreated animals, it was just about the same in the mitochondria and the small granule fraction. An interesting experiment that we did at one time, as a control actually, was to incubate some boiled liver slices with thyroxin, and we found that about 95% of the activity was bound to the boiled tissue. I wonder if this has any bearing on these site binding experiments

DR. LARDY: We never tried boiling the mitochondria, but thyroxin would probably like them, boiled or not.

DR. GROSS: It would probably plaster onto any protein substance available to it. I doubt whether there is any specificity to this action.

DR LEVINE. It was reported recently from Lippman's laboratory that there was a relationship of magnesium to the phosphorylative capacity, and to the action of thyroxin (9). This would indi-

[a] LIPNER, H J, BARKER, S B, and WINNICK, T *Endocrinology, 51*.406, 1952
[b] LEE, N D, and WILLIAMS, R H *Endocrinology, 54* 5, 1954
[c] CARR, E A, JR., and RIGGS, D S *Biochem J, 54* 217, 1953.
[9] LIPMAN. Proc *Nat Acad Sc*, 1955

co-enzyme A. Succinyl co-enzyme A is a nice agent to activate certain fatty acids. Perhaps fatty acid co-enzyme A derivatives could be used to activate amino acids under some circumstances. It may be that the mere presence of fat, or the continual metabolism of fat keeps up the concentration of some enzyme involved in the activation of carboxyl compounds.

DR. ROBERTS: Isn't there a third possibility also? The increased availability or increased oxidative utilization of any substrate, at least when phosphorylation is not simultaneously uncoupled, would indirectly provide energy for protein synthesis. Thus, the utilization of fat which seems to accompany say the action of growth hormone may be explained on this basis. In a similar fashion, returning to Dr. Astwood's remarks, the increased utilization or availability of carbohydrate in the uterus under the influence of estrogens could also be interpreted as providing additional energy, which secondarily results in growth. There need not be any specific or direct stimulation of the processes intimately involved in peptide bond synthesis.

DR. LARDY: Dr. Randle, was adequate carbohydrate present?

DR. RANDLE: I think this phenomenon was seen only with restricted food intake.

DR. LARDY: If that is the case, Dr. Roberts' remarks are very apropos.

DR. GRIFFITH: Going back to the effect of the thyroid hormone and the experiments in which the mitochondria were involved, this morning we heard the term site-binding used frequently. Does the term have significance as regards various parts of the mitochondrial surface, and possible surface effects of the thyroid hormone?

DR. LARDY: Very early in our work, Mrs. Maley reported at a Federation meeting in 1952 that thyroxin and triiodothyronine

are bound to the mitochondria, and they are bound very tenaciously. After the first wash, only a trace of radioactive thyroxin or triiodothyronine can be removed from the mitochondria in a salt solution, which will displace anything that is bound ionically. Hoch and Lippman have since published in one of their papers similar results. In our case the total amount of material bound was far in excess of what one would ever expect to encounter under physiological conditions. I think the binding must be physiologically nonspecific.

DR. GROSS: In this connection there are some data on the distribution of I^{131} on cellular particulates in the liver after radioactive thyroxin administration (a, b). As I recall, with the labelled material there is somewhat less in the mitochondria than in other fractions, and in the case of Riggs' (c) data on the protein bound iodine of these fractions in untreated animals, it was just about the same in the mitochondria and the small granule fraction. An interesting experiment that we did at one time, as a control actually, was to incubate some boiled liver slices with thyroxin, and we found that about 95% of the activity was bound to the boiled tissue. I wonder if this has any bearing on these site binding experiments.

DR. LARDY: We never tried boiling the mitochondria, but thyroxin would probably like them, boiled or not.

DR. GROSS: It would probably plaster onto any protein substance available to it. I doubt whether there is any specificity to this action.

DR. LEVINE: It was reported recently from Lippman's laboratory that there was a relationship of magnesium to the phosphorylative capacity, and to the action of thyroxin (9). This would indi-

[a] LIPNER, H J, BARKER, S B, and WINNICK, T. *Endocrinology, 51* 406, 1952.
[b] LEE, N D, and WILLIAMS, R H *Endocrinology, 54* 5, 1954
[c] CARR, E A, JR., and RIGGS, D S *Biochem J, 54* 217, 1953.
[9] LIPMAN Proc *Nat Acad Sc*, 1955

cate that the effect might be exerted by a chelating action on magnesium. Do you think that this has physiological significance, in explaining at least partially the mechanism of action, and would that indicate that any agent which has the capacity of partially binding magnesium might be capable of doing the same thing?

DR. LARDY: Well, there are two aspects to that question. One is the question of whether thyroxin occurs as a chelate with metals. In the Brookhaven symposium it was pointed out that undoubtedly under physiological conditions, all thyroxin, or virtually all thyroxin, would be chelated with magnesium. We have measured that quantitatively, and have isolated in pure form the magnesium-thyroxin chelate. It contains three molecules of thyroxin per mole of magnesium, and it has a solubility product of 10^{-17}. This is a low solubility product but it is deceiving because it involves a cube factor of the thyroxin, which is present in three equivalents per equivalent of magnesium. But it is also true for most chelates, that the first organic molecule binds more tenaciously than the second one, the second binding more firmly than the third. So, only a very, very minute amount of thyroxin could be expected to be present in tissues in an unbound form. Thyroxin in its active form is probably bound to some tissue component, as well as with diavalent caton, but that does not necessarily mean that it is acting *in vitro* by removing magnesium from the system, because in all systems that we have used, magnesium has been present in a tremendous excess in relation to thyroxin. Therefore, the effect of thyroxin would not be by a mass action removal of magnesium.

DR. LEVINE: In that connection, Dr. Lardy, did not Lippman reverse the action of thyroxin by just adding magnesium?

DR. LARDY: Yes, I think that is a matter of exceeding the solubility product and precipitating out the thyroxin, making it not available to the enzyme system.

DR. BEST: Dr. Levine, would you clarify the action of epinephrine and insulin on plasma inorganic phosphate?

DR. LEVINE: The fall in inorganic phosphate in the plasma after the injection of epinephrine is not due to a direct action of epinephrine, since it occurs in the normal animal, but does not occur in the absence of the pancreas. Insulin causes a fall in inorganic phosphate in the serum; so does glucose in the presence of the pancreas, or when given in very high amounts to the depancreatized animal. Epinephrine will do this in the normal animal, but not in the depancreatized animal. Epinephrine however, will increase the hexose phosphate in muscle even in the absence of insulin. We concluded (10) that the effect of epinephrine on serum P was mediated by insulin, while the effect of insulin on hexose phosphate in muscle was mediated by epinephrine.

DR. RANDLE: It does not fit with the observations of Groen and Willebrands to which I have referred previously. They reported that insulin had no influence on the uptake of inorganic phosphate by diaphragm. I do not think that the influence of epinephrine on uptake of inorganic phosphate by diaphragm has been studied.

There have certainly been studies of the influence of epinephrine on the glucose uptake by diaphragm. Epinephrine, *in vitro* has been reported to inhibit glucose uptake (Walaas, O. and Walaas, E.: *187*:769, 1950), and to antagonize the action of insulin on the glucose uptake by diaphragm (Riesser, O.: *Biochem. & Biophys. Acta., 1*:208, 1947). In the presenc of 8-hydroxyquinoline, Sutherland states that epinephrine will increase the uptake of glucose by the isolated rat diaphragm (Sutherland, E. W.: *Phosphorus Metabolism*. Baltimore, Johns Hopkins Press, *2*:577, 1952).

DR. LEVINE Does it do so in the absence of the liver?

DR. BEST: I'm not sure. I think the experiment has been done with negative results, but I can not name the investigators.

DR. LARDY: In 1936, Dr. Barker reported that epinephrine had no stimulatory effect on oxygen uptake in the rat which was thyroidectomized, and as a matter of fact he found a decrease in

[10] SOSKIN, S., LEVINE, R AND HECHTER, O: 1941. *Am J. Physiol*, *134*:40, 1941.

the oxygen consumption following injection of epinephrine. Dr. Doisy in our department has reinvestigated this, and has found that epinephrine does stimulate oxygen uptake in the thyroidectomized rat, but the effect is much more evanescent than in the normal. There is an effect at about half an hour to an hour, but by two hours the effect is entirely gone. It happened that Barker began his measurements of oxygen uptake two hours after the epinephrine had been administered. So, thyroid hormone augments, or potentiates, or shall we say, reinforces the action of epinephrine, but it is not obligatorily essential for the expression of epinephrine's effect on basal metabolism.

DR. GROSS: Would you like to explain the enhancement of this calorigenic effect of epinephrine by thyroid hormone on the basis of the uncoupling mechanism?

DR. LARDY: No. I have no explanation for it. It might be easy to do that if we knew how either one of them acted, but since they are both in question, there is no point in compounding hypotheses.

DR. GROSS: Here you suggest that there is a direct effect of thyroid hormone on a point in a sequence of oxidations, and then you mention experiments in which virtually all the calorigenic effects are attributable to, or potentiated by epinephrine. Don't you think then that the calorigenic effect of the thyroid hormone may be indirect, rather than direct?

DR. LARDY: Well, there are certainly some experiments to indicate this. The experiments of Brewster, for example, reported at the Federation Meeting two years ago, indicate that in the animal with complete sympathetic block, thyroxin is without effect on oxygen uptake. There are other experiments which have indicated this previously, but I think that with all the hormones, we must consider Ingle's "permissive action." Epinephrine appears to act "permissively" with respect to the thyroid hormone, and the thyroid hormone may act somewhat "permissively," with respect to epinephrine. We are coming back to Dr. Levine's con-

cept that all hormones are interrelated, and you can't isolate the effect of any one of them unless you have all the others there.

DR. KINSELL: At this point, do you suppose that any one would have any thoughts regarding the sulfonamide "insulin-sparers?" This has not been included as official material for the conference, but I think some of the members here have some data. It may not be unrelated to the general philosophy of the program.

DR. BEST: A great many laboratories are working on this problem of various sulfonamides that have an adjuvant action to insulin. Dr. Levine has done a great deal of work in the last two months. The Lilly Research Laboratories held a conference a little while ago, and all of the known work was reviewed. I believe that there is to be another one in a few weeks' time, with representatives of the German workers in this field. I think everyone is agreed that none of these materials function in the complete absence of the pancreas. Initially, there was some confusion about this, but that probably was related to the fact that some insulin is left in the body of an animal for four or five days. This may be related to Dr. Stadie's phenomenon of insulin binding. Dr. Levine told me today that his experiments showed that BZ-55 has no action in the eviscerated animal. There is no doubt about its effect in implementing the action of insulin in producing hypoglycemia in normal animals, including man, and I think there is little doubt that it may supplement the action of exogenous insulin in the depancreatized animal. The hunt for the exact mechanism is probably as broad as the search for the mechanism of insulin I gave a talk on the possible mechanisms of these effects the other day in Dallas, and used Dr. Stadie's slide on the locus of insulin action as an indication of some of the mechanisms which might be affected. Having lived through a period in which hundreds of alleged adjuvants have been tested, and so many of them found to have a toxic action on the liver, one always has this point in mind The great interest in this substance, BZ-55, and certain other sulfonamides is that they have been given so long to human beings without evidence of toxicity. Perhaps, however, we have to go on much longer in human subjects and conduct very long

term animal experiments to prove that these substances are free of any poisonous action on the liver. There have been reports that the action of insulinase is inhibited by this substance. I have been telephoned in Toronto by firms in this country stating that their particular substance is the best inhibitor of insulinase. This is very interesting but there are of course, many chemicals which may inhibit insulinase *in vitro*, which would not be acceptable therapeutic agents. One of the most interesting statements that I have heard recently is the one I quoted from Dr. Levine. Perhaps he would like to amplify this. Dr. Mirsky's paper on insulinase inhibitors has now appeared in "Metabolism."

DR. LEVINE: We have been using this material both in patients and in animals, and while our results of course are very sketchy, they are sufficient to indicate the following: It lowers the blood sugar in normal animals and in normal humans at a rate that is not too impressive unless you give the sodium salt. Since in that form it is absorbed more quickly, there is a faster fall. In animals, pancreatectomy abolishes the action; so does evisceration. It acts very quickly, and brings the blood sugar down almost to zero in the adrenalectomized rat, with pancreas intact; probably because the animal is very insulin sensitive. In human diabetics we have given two grams of the sulfonamide in the morning, withdrawing the insulin dose that morning The blood sugar falls to less than 50% of the initial value in four to five hours. Many adult diabetics, irrespective of the duration of the diabetes from the time of the first diagnosis, can be managed on the drug with good control. In the juvenile diabetics, the blood sugar does not fall significantly. In five patients on extended study, we have done liver function tests, hemograms, microscopic and chemical analysis of urine, blood electrolytes, proteins (the usual studies for toxicity) as well as I^{131} uptake, PBI^{131} and chemical PBI, because the substances are sulfonamides, and we remembered Dr. Astwood's and Dr. Mackenzie's work. Thus far there does not seem to be any change in the liver function studies, no changes in the red blood count, the white blood count, and no crystalluria. In two patients there was a moderate drop in the iodine uptake, and this occurred on small doses of one or two grams daily. What will hap-

pen on long term administration we do not know. I would like to emphasize what Dr. Best said, that any substance, no matter how effective, that is designed to substitute for insulin must be a substance which, if we have to give it for 30 or 40 years, should not be toxic. We can't run experiments for 30 or 40 years, but it seems to me that we must have more data over an extended period of time before decisions can be reached.

As to mechanism, we have not tested insulinase inhibition directly. Mirsky has studied two sulfonamides and has showed that they inhibit insulinase *in vitro*, but whether they would inhibit other proteolytic systems as well, or other enzymes which are not proteolytic, is not known. I have begun the use of sulfonamides in some selected juvenile diabetics, not by withholding all insulin, but attempting to reduce the insulin, and seeing whether the addition of BZ-55 would smooth the course of such a brittle diabetic. It is not as yet clear whether in addition to the inhibition of insulinase, there is another effect. Does this material stimulate the secretion of stored insulin from Beta cells? Is that its primary action? What we would like to see therefore, are careful studies on the I.V. injection of insulin with and without BZ-55 in the depancreatized animal and in the normal animal. Since De Duve has shown that glucagon seems to be hydrolyzed in the liver by the same enzyme system, i e., insulinase, is there a possibility that at certain dose levels one may inhibit the destruction of glucagon as well as of insulin?

DR. LARDY BZ-55 is sulfonamide-urea-butyric acid. U-2043 has a methyl instead of the amino group on the ring. BZ-55 is a rather good antibiotic. I am told that the methyl compound has little antibiotic potency.

DR. RANDLE· There is a third class of compounds—the alkyl thiodiazole derivatives of sulphonilamide. These were extensively studied by Loubatieres and shown to be hypoglycemic (Loubatieres, A. *Compt Rend. Soc. Biol.* Paris, *138*:766, 830, 1944, *Arch. Int Physiol*, *54*:174, 1946). Their hypoglycemic action was first described by Janbon and his associates (Janbon, M, Lazerges, P. and Metropolitanski, J. H.: *Montpellier Med. 21-22*:489, 1942).

Loubatieres thought that the sulphonamide derivatives evoked hypoglycemia by stimulating insulin secretion.

Another possible mechanism of action of sulphonamide derivatives which hasn't been discussed is the suggestion by Holt and his colleagues that they inhibit glucagon secretion by selectively damaging the cells of the pancreatic islets (Holt, C., Holt, L., Kroner, B. and Kuhnau, J.: *Naturwissenschaften, 41*:166, 1954). It has been claimed, but not confirmed, that the blood sugar of alloxan diabetic rabbits could be normalized by the administration of sulphonamide derivatives—virtually isletless animals could have normal blood sugar levels (Holt, *et al.*: *Naturwissenschaften, 41*:166, 1954). Have you studied any effects of sulphonamides on pancreatic histology, Dr. Best?

DR. BEST: Dr. R. E. Haist is in process of doing this. The destruction of alpha cells does not appear prominently. I believe some work has been done in the Lilly Laboratories which does not indicate alpha cell destruction.

DR. LEVINE: An alloxanized animal with severe diabetes—one in which you are sure that the beta cells are gone, does not respond to this material with hypoglycemia.

DR. RANDLE: But if this substance doesn't act in the hepatectomized animal, and potentiates the action of insulin or stimulates the secretion of insulin, then presumably insulin must have an action on the liver.

DR. LEVINE: I should not have said the hepatectomized animal, I should have said the eviscerated animal. Both the liver and the pancreas were removed.

DR. HOUSSAY: The hypoglycemic actions of sulphonamides was studied first by Loubatieres from 1942 until today. He used the para-amino-benzene-sulfamido-isopropyl-thiodiazol (2254 Rhone Roulanc) and Bovet et Bubost (1944) and Loubatieres (1944-46) could demonstrate that the lateral chain tertio-butyl, isobutyl, amyl and iso-amyl, propyl and iso-butyl are also active. The action

was only present in animals with pancreas, not those without pancreas, and we have confirmed this result. The action is very intense when injecting the substance by the pancreatic vein or the pancreatic duct. I remember that in 1946 we found with Martinez that many substances can increase the free SH content of tissues (cysteine, thiouracil). There is a marked protection against the toxic and diabetogenic action of alloxan. Thyroidectomy also gave protection, but the consecutive treatment with thiouracils increases the protection. We find also some preventive action on the frequency of diabetes that develops after large subtotal pancreatectomy. Dr. Lott, of Squibb and Sons, was kind enough to prepare these substances with Dr. Yale. One was the 5 isopropyliden 2,4-dithiohydantoine. Dr. Williams, of the University of Washington in Seattle, has found that this substance produces a slower disappearance of insulin in the blood. We don't know whether the hypoglycemic sulphonamides have an action on the liver or not. It would be important to study the action of these substances on thyroid function, because some sulphonamides have this action. In our experiments many substances with protective action on rat diabetes were also goitrogenic. Hypothyroidism has a protective action on diabetes in the subtotally pancreatectomized rats. Thiouracil ameliorates the diabetes of thyroidectomized-pancreatectomized rats, hence it has an extrathyroidal action.

DR. WILLIAMS: As Dr. Houssay has said, we have carried out a number of studies with one compound in particular that he studied, 5-isopropylidene-2, 4-dithiohydantoin, Squibb product MC-2346. This compound in *in vitro* studies will cause more than 90% inhibition of the degradation of insulin-I^{131}. BZ-55 and D-860 also cause significant inhibition. As Dr. Houssay says, we find that when we give MC-2346 to rats *in vivo*, we can show that there is a slower degradation of insulin as evidenced by a greater amount of trichloracetic acid precipitable material in the blood and in various tissues We recently have carried out a study in which we have shown that if we do a nephrectomy (it was previously shown that nephrectomy decreases the degradation of insulin) that the use of MC-2346 has no demonstrable effect. That is, the amount of degradation in a nephrectomized animal is the same whether

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diabetic in the middle-aged group, who may be obese (but not necessarily so), the fall in blood sugar in 4 hours may be from about 200-250 mg. to 100-140 mg. or 40 to 50%. At the end of 8 hours, the blood sugar may have risen a little. During this period the blood sulfa rises to values ranging between 6 and 8 mg. In one woman, admitted to the hospital for an infected toe, who had been taking insulin for several years, up to 40 units, BZ-55 was used with a complete replacement of insulin. We did not suspect hyperthyroidism at first. After she had received BZ-55 in doses up to 3.0 grams a day for several days, the radioactive iodine uptake was at a diagnostic level of plus 60%. We then omitted BZ-55 and some days later the radio-active iodine uptake had risen to 75%. She was then put on treatment for hyperthyroidism. Whether the drug had had any effect on depressing the first reading may be questionable, but of some interest in connection with other observations of the effect of BZ-55 upon thyroid function. Another woman who had been insulin-resistant for some years and had taken since the age of 63 years from 150-240 units of regular (U500) insulin daily entered the hospital because she had been found unconscious after a fall. The possibility of an insulin reaction was considered but ruled out. Gradually, we were able to replace the insulin entirely with BZ-55. At the end of the period, while the urine was not sugar-free, her blood sugar levels were about at the same point as when she had been on the dose of 140 units of regular insulin. She finally left the hospital to go to a nursing home. She had been returned to regular insulin, but was taking only a fraction of the dose she had formerly required.

DR. KINSELL: We have a few studies which have been carried out, for the most part, in unstable juvenile diabetics. In two such patients, on a dosage considerably larger than has been used in the middle-aged diabetics, there was a very striking decrease in 24 hour iodine uptake by the thyroid, to levels below 5 percent. The change appears to be transient. These are very current observations, so we have no real concept of how uniform an effect this is, and how it may or may not be related to dosage, and what the average duration of this inhibition of uptake may be. In one juvenile diabetic on large dosage and maintained on chemically constant food intake, there was accentuation of hyperglycemia and

you do or do not give MC-2346. Incidentally, we do know that MC-2346 produces morphological damage of the kidneys in some instances.

I would also like to comment on some of the other subjects that have been discussed. In keeping with Dr. Levine's comment, we have used many different approaches and have repeatedly come to the conclusion that so-called "insulinase" also degrades glucagon, degrades alpha-corticotropin, and degrades casein. In line with these observations Dr. Tyberghein working in our laboratory, has recently uncovered some interesting information which I think helps to explain some of the confusion that has existed with respect to the action of glucagon. He used an amount of glucagon that is so minute when added to slices of liver that no glycogenolysis took place. Novo insulin, which of course is free of glucagon, produced no glycogenolysis when used alone. The combination of this minute amount of glucagon plus the Novo insulin caused a marked glycogenolysis. We interpreted these observations as indicating that the insulin serves as a substrate competitor, and you might say, affords "insulinase" something to work on, thereby permitting glucagon to carry out its action. We get essentially the same effect using insulin reduced with cysteine. Back in 1946 there was a paper in the *Proc. Soc. Exper. Biol & Med.* by Chen *et al* reporting that 2-sulfanilamido 5-cyclopropyl-1, 3,4-thiodiazol damaged the alpha cells. When it was given to severely alloxanized animals, it produced hyperglycemia. This could be attributed to an inhibitory effect of the sulfonamide on the enzymatic degradation of glucagon. In a similar manner BZ-55 may increase the effectiveness of insulin. Bertram and his colleagues, in a group of papers in *Klinical Wochenschrift*, reported damage to the alpha cell in about half of their rabbits, but they used huge doses. I am inclined to think that the changes in the alpha cells result from hypoglycemia rather than cause it.

DR. ROOT: We have been using BZ-55 in a series of 40 or 50 diabetic patients Our experiences with its rate of action are as already described. We have given the substance to new patients who have never before had any insulin and we have also given it to other patients who had been on moderate to large doses of insulin for various periods of time. In a typical newly discovered

I would also like to say that Dr. Tyberghein, working in our laboratory with liver slices, finds that D-860 partially inhibits glycogenolysis.

DR. BEST: I would not think that glucagon could produce an intense diabetes just by an intense glycogenolysis alone. The nitrogen excretion of these animals is doubled within twenty-four hours, so I would anticipate another action of glucagon, in addition to that on glycogen. There is a possibility that there is more than one substance in the glucagon we have used. Most of the glucagon is only about 50% pure. There is very little of the crystalline substance available, and Salter and Davidson have only made preliminary studies with this material. The crystalline material does however, give the typical diabetogenic effects.

DR. LEVINE: Dr. Best, can you produce a "metaglucagon diabetes?"

DR. BEST: We haven't accomplished that yet. There are a lot of different effects. The appetite is affected and we have to force feed to get the diabetes. There is not the clean cut picture of a beta cell necrosis that you get in dogs with, say growth hormone. It is a much more complicated picture and we are prepared to find some other mechanism operating here.

DR. LARDY: I would like to ask one question of the people who have worked with the sulfa compounds, and that is, is there any evidence that they would inhibit glucose-6-phosphatase? This would be a possible way of action.

DR. WILLIAMS: That was one of the first things that we thought about, but when we observed that under certain conditions the responses to glucagon were normal we discounted this. However, recently we have shown that D-860 partially inhibits glycogenolysis and glucose-6-phosphatase.

DR. LEVINE: The fact is that this material when given to a diabetic brings the blood sugar down (in some diabetics to normal). The glucose tolerance definitely improves. Since that is so, and the major action is in the periphery, then the action is probably not on glucose-6-phosphatase.

glycosuria, the glycosuria at one period approaching the entire amount of intake of carbohydrate by mouth. Despite this, there was essentially no hyperketonemia. In another juvenile diabetic, after a brief period of little or no apparent effect, there was a rapid and major decrease in urinary and blood glucose. Both of these patients received constant daily insulin, but at a dose level purposely set well below their usual requirements. All of this perhaps suggests some type of insulin sparing effect.

DR. BEST: Dr. Stadie said that BZ-55 had no effect on the glucose uptake of the rat diaphragm. Did you try with a certain dose of insulin to see if BZ-55 supplemented the action of insulin? And Dr. Levine, I think you found no effect on the disappearance of galactose. Did you try the supplementary effect of BZ-55 with standard doses of insulin?

DR. LEVINE: We did it with a small amount of insulin and didn't see any effect. We are now going to try graded doses.

DR. BEST: Dr. Williams, a few months ago, I wouldn't have expected a decreased secretion of glucagon to have much effect on the diabetic state, but as I have stated large doses of glucagon in oil are intensely diabetogenic in rats. The amount we used may be far outside the physiologic range. This effect may be due to some quite bizarre phenomenon affecting the beta cells. We haven't had time as yet to do complete histologic studies. Glucagon might be an antimetabolite of insulin and in some way interfere with the formation of the latter.

DR. WILLIAMS: In regard to Dr. Best's comment, I would like to state that I have thought for a long time that if there was a way of getting a continuous action of glucagon, such as by giving a long acting glucagon preparation, it might be possible to produce diabetes. Earlier attempts by Root to produce diabetes by glucagon apparently failed because the injections consisted of only very short acting material given only once or twice a day. Lukens and others have shown that a hyperglycemic state maintained for several days by glucose administration will produce sustained diabetes.

Dyke have found this action in an impure "erythropoietic agent" that they have identified in the pituitary gland. These are not pure substances. We know that there is something else in the extract, but we do not know what it is. I will not try to discuss this problem further. Instead I will pass to carbohydrate metabolism.

These divisions of metabolism into carbohydrate, protein, and fat are completely artificial because such processes are all connected. The first fact in regard to carbohydrate metabolism is that in the hypophysectomized animal there is slow intestinal absorption of sugar and some other substances. This is due chiefly to diminution in thyroid function, and can be corrected by giving thyroid hormone. But the most typical change after hypophysectomy in animals is the inability to maintain the carbohydrate levels in the body during fasting. The animal, when fasting, after a few hours develops hypoglycemia, and a decrease of glycogen in the liver, muscle and heart. This decrease in glucose and glycogen can be prevented by feeding carbohydrate or protein, but not fat. The administration of some hormones, somatotropin particularly, and ACTH or cortical hormones in part, can prevent some of these changes. Somatotropin prevents especially the decrease of glycogen in muscle. Very striking is the decrease in blood sugar in these animals during fasting and during the administration of hypoglycemic agents, such as insulin or phloridzin. These animals have hypoglycemic symptoms and coma, and will die if they are not treated with sugar. The remarkable sensitivity to insulin was the first fact that put me on the track of the importance of the anterior pituitary in carbohydrate metabolism back in 1924. The hypophysectomized animal is much more sensitive to insulin than is the adrenalectomized animal or thyroidectomized animal. The sensitivity is due to suppression of the pars distallis, or the anterior lobe. It can be corrected by giving anterior lobe extracts. That was also the first indication we had (Houssay and Rotick, 1929) that the anterior lobe was important in pituitary regulation of metabolism. This hypersensitivity to insulin can be corrected by somatotropin, ACTH, prolactin and by corticoids. Another interesting fact is that the animal with hypophysectomy has less hyperglycemia than the normal, when treated by hyperglycemic agents. This depends on the substance used, adrenalin, pilocar-

III

THE ANTERIOR PITUITARY IN RELATION TO ENERGY METABOLISM

By Bernardo Houssay

The anterior pituitary gland has a regulatory action on most of the functions of the body. The physiological role of the anterior pituitary is very important, and is necessary for the maintenance of the normal endocrine and metabolic state of the individual, and also, due to its action in reproduction, this gland is important in the maintenance of the species. The principal action of the pituitary is one of regulation of metabolic functions. In some cases the action is specific on certain target organs. The actions of the hormones of the anterior pituitary are sometimes direct, as in the case of somatotropin or prolactin. In some other cases the action is mediated by some other gland, such as the thyroid, adrenal and gonads.

There is an action of the pituitary on energy metabolism, heat production, and oxygen consumption. By the action of thyrotropin on the production of thyroid hormone secretion the pituitary has regulatory importance on these phenomena. After hypophysectomy there is diminution of thyroid function and diminution of basal metabolism, but not complete supression of thyroid function. Thyroidectomy produces a further decrease of metabolism in hypophysectomized dogs, but hypophysectomy does not decrease the metabolism of thyroidectomized dogs. Thyrotropin or total pituitary extract injections increases metabolism, calorigenesis and oxygen consumption. This is due principally to thyroid stimulation, because the action of thyrotropin is not present in thyroidectomized dogs, and the action of pituitary extract is diminished. It is not known what are the pituitary substances, outside of thyrotropin, that can produce extrathyroidal metabolic stimulation. Collip and his callaborators have described one active agent, not isolated to date. Consopoulos, Ellis, Simpson and Van

Bodo. Gluconeogenesis also is probably below normal. So far as peripheral action is concerned, I shall pass over it for the present, for probably after the paper of Dr. Randle we can discuss the action of pituitary and insulin on the peripheral tissues.

Pituitary extract or pituitary hormones can correct the deficiencies I have mentioned. Such treatment abolishes the fasting hypoglycemia, *and the diminution of glycogen characteristic of* the hypophysectomized animal. The diabetogenic action is easily obtained in hypophysectomized depancreatized animals. This is also true in the animal with partial pancreatectomy. The easiest way to sensitize an animal to the diabetogenic action of the hypophysis is to reduce the mass of the pancreas. Then, leaving a small part of pancreas, sufficient to maintain normal glycemia, the animal manifests 100 to 500 times more sensitivity to pituitary hormones than the normal. The depancreatectomized dog injected with pituitary hormones develops ketonuria and acidosis in one, two, or three days. There is then an extra-pancreatic action of pituitary somatotropin that becomes accentuated in animals without a pancreas. So the principal action is extra-pancreatic.

In normal animals the diabetogenic effect was demonstrated in 1932 by three laboratories at the same time. The first publication came from Evan's group, with whom I was in correspondence at the time. Since then this action of diabetogenesis has been studied in many animals. There is often a lag of one, two or three days before the action occurs. Glycogen does not diminish except when there is severe diabetes. There is great resistance to insulin, hyperglycemia, and glycosuria and the glucose tolerance curve is of a diabetic type. After giving sugar the respiratory quotient usually does not rise, and all the typical symptoms and signs of diabetes appear. If the treatment is maintained for some days and then the injections are stopped (using crude extract or somatotropin), the blood sugar returns to normal. This takes one, two or three days, and the islets of Langerhans, if lesions were present, return slowly to normal in five or 10 days. This is hypophyseal diabetes of a type which Dr. Young prefers to call idiohypophyseal diabetes. The first phenomenon in animals so treated is, first, an increase in resistance to insulin. There are no visible lesions in the beta cells. The primary action is probably extra-pancreatic as

pine, or ether. Adrenalin was shown to be less active by Asher a long time ago, in 1912, and confirmed by one of my collaborators, Braier in 1931. A remarkable fact is that sometimes when giving insulin or other substances, rapid mobilization of glycogen is not seen. Sometimes, when rapidly fatal insulin hypoglycemia is produced in these animals, at autopsy we are surprised to find high liver glycogen.

Another fact that is classical now is the attenuation of diabetes by hypophysectomy. At first we did this in dogs and toads with pancreatic and phloridzin diabetes, but afterwards we have done it with all types of diabetes. This includes meta-alloxanic, metathyroid, metacorticoid, and metahypophyseal diabetes. In all these cases, attenuation of diabetes can be produced by removal of the anterior lobe. There is diminution of hyperglycemia and glycosuria. In the post-absorptive period there is less hyperglycemia, and if these animals are fasted they will develop hypoglycemic symptoms and die unless they are given sugar. They manifest a striking sensitivity to insulin. After administration of glucose they do not excrete it all, even by the next day. Most striking is the great diminution of ketone body excretion. Similar phenomena have been demonstrated in isolated tissues by Stadie. Dogs and cats with pancreatectomy have greater glycosuria and hyperglycemia than the same animals following pancreatectomy and hypophysectomy. In the cat, the nitrogen excretion is diminished, and the loss of body weight is strikingly less; the animals survive a long time in fair condition. In isolated tissues the consumption of glucose by isolated heart muscle is higher following both hypophysectomy and pancreatectomy (Shorr). Deamination and ketogenesis in liver slices from animals without hypophysectomy are normal, and lipogenesis from acetate is also present. Concerning the mechanism of hypoglycemia in these animals in response to fasting or insulin, there appear to be two important facts. One is that there is supernormal glucose consumption, as demonstrated by Fisher, Russell and Cori in rats, and also by Greeley in eviscerated rabbits. But the principal reason is that in the normal liver the hypoglycemia increases the production of glucose, whereas in the hypophysectomized animals there is none. This has been demonstrated by Crandall and Cherry and also recently by De

I will not say very much about adreno-corticotropin and about the role of adrenal. The presence of cortical hormones is very important for the complete diabetogenic action of pituitary extract or somatotropin. In the first experiment of Long and Lukens they reported that pituitary extract was completely devoid of action in the absence of the adrenals. In our experiments in toads and dogs we were able to demonstrate that there was marked diminution, but not complete absence of action in the adrenalectomized animal. We obtained an intense diabetogenic action of somatotropin and prolactin in dogs with 80% of the pancreas removed, and without either hypophysis or adrenals.

Our experience agrees with that of Long and Engel and their respective groups that adrenal cortical hormones and pituitary hormones have synergistic action. In other words, with the two substances given together the action is more striking than with either one alone. The only exceptions are found in some experiments by De Bodo in hypophysectomized animals. I have discussed this with De Bodo and he believes that perhaps the action is different in the hypophysectomized than in the normal animal. I will not discuss it further since De Bodo is not here, and perhaps he has not published all the facts yet. However, he found that in the hypophysectomized animal when he tried to normalize the blood sugar by somatotropin alone, it was impossible, because the animals developed increased resistance to insulin, or diabetes, but on giving corticoids together with somatotropin, it was possible to obtain a normalization of carbohydrate metabolism. These observations need to be studied further.

Concerning insulin and pituitary extract, there is usually some antagonism demonstrable. For instance by removing the pituitary the animal becomes sensitive to insulin, and by taking away the pancreas the animal is made sensitive to pituitary. In many instances there is antagonism but not always. A very typical case in which there is no antagonism is in growth, in which it is not possible to obtain the action of growth hormone without insulin. That was first demonstrated by Mirsky and later by Lukens. Also it is not possible to obtain protein anabolism from somatotropin in the absence of insulin. In rats there is some possible contradictory evidence in that rats made diabetic with alloxan sometimes show some growth or protein anabolism by the action of

this phenomenon is obtained also in animals without a pancreas. During this period, the content of insulin is not diminished in the pancreas, secretion of insulin is good and the gland appears at first not to be severely involved. But after some days lesions may be seen in the beta cells, the so-called degranulation and hydropic degeneration, with glycogen infiltration. Then after some days there is pyknosis in some of the nuclei, and progressive decrease of the beta cells. If the injection is prolonged for many days the animal has permanent diabetes. We obtained permanent diabetes in 1932 by injecting partially pancreatectomized animals. Young did the same in animals with total pancreatectomy, in 1937. He has extensively studied this type of animal, and for this reason Loubatieres has called this type of diabetes "Young Diabetes." It is remarkable that under some conditions at the beginning, pituitary extract can produce a diminution in blood sugar. Probably Dr. Randle will speak of that. During hypophyseal and metahypophyseal diabetes the phenomena in the pancreas involve anatomic alteration, and diminution of insulin production.

What are the hormones involved in this action? Using purified hormones in dogs and cats, the most active is somatotropin. But species specificity is important. In some animals (rats) adrenocorticotropic hormone is more active; in some (dogs) prolactin is more active than ACTH. The great problem is to know whether the action is real due to the specific hormones or to some impurity remaining from their preparation. In some experiments Dr. Raben, working in the laboratories of Dr. Astwood has found one preparation of somatotropin with growth action but no diabetogenic effect. Dr. Raben sent me a sample of that preparation and we obtained diabetes. Up until now there is no clear demonstration that there are interfering substances in the preparations of somatotropin In the case of prolactin, preparations made by different persons have had variable activity but very purified prolactin has had less potency. For instance Dr. Li sent me some samples of prolactin, and they had less diabetogenic action than those prepared by Dr. Bates. The action was clearly not due either to somatotropin or to adreno-corticotropin. The fact that there is no parallelism between the specific action of prolactin and its diabetogenic effect suggests that the latter may not be attributed to the specific hormone itself.

betes, as for example in acromegaly. In the book of Atkinson who has collected all of the papers on acromegaly in the world literature, there are 817 cases recorded. He found that diabetes occurred in 32%. Dr. Root published, in 1943, a series of acromegalics in which he found the incidence of diabetes as 19%, and 36% of the cases had occasional glycosuria. There are also some children who show accelerated growth during the period when diabetes appears. I will not discuss this problem further.

On protein metabolism, just a few words. In the animal without the hypophysis, protein anabolism is usually diminished. In the rat which has anorexia, usually there is a decrease in nitrogen retention. Dr. Samuels with Reineka and Baumann have demonstrated very clearly that if hypophysectomized rats receive forced feeding in sufficient quantity with sufficient amounts of protein, they can respond with increase in weight, some increase in protein storage, and real growth. The formation of protein then is less easily obtained, but is still possible. Dr. Scow also has experiments of this type. There is less growth and if the animal receives a high caloric diet, there is adiposity. The reason is not clear and it is a problem that needs to be studied. One reason obviously is that they eat a high caloric diet in relation to their metabolic requirements but undoubtedly there are some other reasons. The animal develops adiposity easily if it is force fed, and although it is possible to obtain a small positive nitrogen balance and increment of growth, it is very difficult.

There is another problem related to the pituitary. As we have seen, an animal without a pituitary has practically no growth. If the animal (dog) receives meat, in 24 hours his urinary nitrogen excretion is the same as in the normal dog (Braier, 1931). Thus the catabolism of the dietary protein is not disturbed and the catabolism of the absorbed amino acids is normal By injecting amino acids the utilization is also normal. De Bodo has similar evidence, with some slight difference in that in the Braier experiments, in the first seven hours there is slightly less urinary excretion of nitrogen but it is normal for the 24 hours. However, when the animal is forced to mobilize his own endogenous protein there is less facility in the hypophysectomized dog. For instance, in fasting, the excretion of nitrogen is two thirds of that of the

growth hormone. However, using totally pancreatectomized animals, there is no growth and no nitrogen retention. Dr. Scow of the National Institutes of Health, in Bethesda, who is now working with me in Buenos Aires, has studied the same phenomenon and has done many experiments. With growth hormone and insulin there is growth of pancreatectomized or pancreatectomized-hypophysectomized rats. If the animals receive somatotropin and no insulin there is no growth or nitrogen retention. With insulin alone there is a little growth, but on receiving insulin and growth hormone together there is typical intense growth. Nitrogen retention is not very great in the depancreatized animal with insulin alone, but much more in the animal with insulin and growth hormone. There is a slight retention in some of the animals which received 12 units per rat, which is a large dose, considered in relation to food intake. Without food they can be killed with a half or a quarter unit of insulin, but by giving food it is possible to inject 15 units and sometimes we have injected up to 20. With reduction of insulin to two units there is no more nitrogen retention, and no more growth. It is remarkable that if we again give insulin there is no immediate nitrogen retention. Only after 10, 12, or 15 days do we again have nitrogen retention. This lag is a very interesting phenomenon which we have been seeing in many animals. By giving more growth hormone there is nitrogen retention while maintaining the same amount of insulin. In these experiments of Dr. Scow in the rat, there is no growth and no nitrogen retention by somatotropin without insulin as Dr. Lukens has observed in cats.

I will not discuss growth and its relation to diabetes but I would like to say a few words about the pituitary and diabetes. We have no evidence that the pituitary produces human diabetes mellitus, but we have shown that the presence of the pituitary gland increased the severity of diabetes mellitus, as indicated by the demonstration that hypophysectomy reduces the intensity of all diabetes. There are publications by Luft and Oliveacrona, and Kinsell and his associates, to the effect that hypophysectomy produces a decrease in severity of human diabetes. Also, in some pathological cases in which there is a clear increase in pituitary growth hormone, there is also some increase in incidence of dia-

IV

THE MECHANISM OF THE INFLUENCE OF PITUITARY GROWTH HORMONE ON METABOLISM

By P. J. Randle and F. G. Young

The administration of pituitary growth hormone to intact rats, cats or dogs leads to a change of metabolism characterized by the deposition of new protein, enhanced catabolism of fat and depressed utilization of carbohydrate. In the dog and cat, but not the rat, the depression of carbohydrate utilization is severe enough to produce diabetes. In the cat and dog, growth hormone still promotes nitrogen retention, at least during the early stages of the induced diabetes, and in this important respect growth hormone diabetes differs from pancreatic diabetes.[1]

Although growth hormone characteristically depresses glucose utilization, and in suitable species produces hyperglycemia, nevertheless the hormone can, in acute experiments in suitable species, produce hypoglycemia and increased utilization of glucose. This latter action of the hormone, which has been termed the insulin-like action of growth hormone[2] contrasts strangely with its better known diabetogenic action

The insulin-like effect of growth hormone and its influence in promoting protein anabolism are both actions of the hormone which appear to depend upon the availability of insulin. It is possible too that species differences with respect to the diabetogenic action of growth hormone may relate to the ease with which insulin is secreted by the pancreas.

Three aspects of the activity of growth hormone have been selected for detailed discussion, namely—the influence of growth hormone on the secretion of insulin by the pancreatic islets; the mechanism of the insulin-like action of growth hormone; and the means by which growth hormone depresses glucose utilization.

control. This was first shown by Aschner (1912) and very well studied by Braier (1931). If the diet is protein free, but otherwise adequate, there is even a greater difference. There is a 40% excretion of nitrogen in the hypophysectomized dog as compared to the normal control. In fasting there is also less excretion of creatinine, purines, phosphate and potassium in the hypophysectomized dogs. In pancreatic diabetes it is very remarkable to see the diminution of excretion of nitrogen in the urine when the dogs are also hypophysectomized. In phloridzin diabetes, in avitaminosis B_1, and after the injection of coli vaccine, the excretion of nitrogen is less in the hypophysectomized dog. Speaking in general terms, it would appear that the mobilization of endogenous protein is diminished in the hypophysectomized dog.

I will not speak further about protein and I will not discuss fat metabolism in relation to pituitary function. I would prefer to wait for the general discussion. Thank you.

islets of the rat. Thus they observed that the administration of insulin led to an increase in the level of glycogen in interscapular adipose tissue, whereas growth hormone was without such an effect. Since growth hormone did not prevent an action of insulin on the glycogen content of interscapular adipose tissue when both hormones were given together, Scott and Engel concluded that growth hormone did not promote the secretion of insulin by the pancreatic islets of the rat.

Milman, de Moor and Lukens[11] obtained indirect evidence which suggested that growth hormone promoted the secretion of insulin by the pancreatic islets of the cat, since they found that in depancreatized cats receiving insulin, treatment with growth hormone promoted nitrogen retention to an extent comparable to that observed in intact cats, only when extra insulin was supplied to depancreatized animals during the period of growth hormone treatment.

We have attempted to study the influence of growth hormone on insulin secretion in the cat and rat by a method of insulin assay based upon the *in vitro* uptake of glucose by the isolated rat diaphragm.[12] This method of insulin assay, a modification of that first described by Groen, Kamminga, Willebrands and Blickman,[13] has been used to study the insulin activity of plasma from rats and cats treated with saline or with growth hormone.[14] Since there is some doubt as to the specificity of this method of insulin assay when applied to the insulin content of blood plasma the term insulin activity of plasma is used without prejudice as to whether the method measures insulin alone in plasma.

With this technique the insulin activity of plasma derived from the femoral vein blood of intact cats treated for five to eight days with growth hormone was found to be significantly greater than that of plasma from control cats which had received saline. This was true irrespective of whether or not the cats developed temporary diabetes as a consequence of the treatment with the hormone. When intact cats were given a single intravenous injection of 2.5 mg. of growth hormone, and blood collected before and five minutes after the injection, no change in the insulin activity of the plasma was detected following the administration of the hormone. Plasma from depancreatized cats deprived of insulin for 48 to 72

GROWTH HORMONE AND THE SECRETION OF INSULIN

Bennett has recently used the pancreatic transplant technique, developed by Houssay, to study the effect of acute growth hormone administration upon the secretion of insulin by the pancreatic islets in the dog. Bennett obtained evidence in this way which suggested that the acute administration of growth hormone led to an increase in the rate of secretion of insulin by the pancreatic islets of the dog.[3]

Anderson and Long[4] have studied the influence of growth hormone added *in vitro*, upon the rate of secretion of insulin by the isolated perfused pancreas of normal fasted rats. Insulin was detected in the perfusate by the blood sugar response of adrenodemedullated, alloxan-diabetic, hypophysectomized rats. Under the conditions of these experiments, growth hormone added *in vitro* to the perfusate appeared to depress the rate of insulin secretion when the level of glucose in the perfusing fluid was abnormally high, but had no detectable influence on insulin secretion when the level of glucose in the perfusing fluid was within the limits of normal fasting blood glucose level. Anderson and Long concluded from these studies that growth hormone suppresses the secretion of insulin which normally occurs as a response to hyperglycemia The validity of such an inference is however doubtful, since there is evidence that growth hormone may promote the release of an hyperglycemic factor from the pancreas[5,6,7] and the presence of such a factor might have interfered with the detection of insulin by the method of assay used by Anderson and Long. Furthermore the studies of Park, Brown, Daughaday, Cornblath and Krahl[8] and of Park and Bornstein[9] have suggested that *in vivo*, growth hormone can be transformed into another molecule with a different action on carbohydrate metabolism and that this transformation depends upon the availability of adrenal steroid. Since the influence of the adrenal was lacking in the *in vitro* preparation of pancreas used by Anderson and Long, the results which they obtained might not be revelant to the influence of growth hormone on insulin secretion *in vivo*.

Scott and Engel[10] failed to obtain evidence for an influence of growth hormone on the secretion of insulin by the pancreatic

islets of the rat. Thus they observed that the administration of insulin led to an increase in the level of glycogen in interscapular adipose tissue, whereas growth hormone was without such an effect. Since growth hormone did not prevent an action of insulin on the glycogen content of interscapular adipose tissue when both hormones were given together, Scott and Engel concluded that growth hormone did not promote the secretion of insulin by the pancreatic islets of the rat.

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hours, possessed no detectable insulin activity. When depancreatized cats were treated with insulin in amount sufficient to restrict glycosuria to 5 to 10 g./day without the development of ketonuria or hypoglycemia, the insulin activity of the plasma was similar to that of plasma from intact cats treated with saline. When depancreatized cats receiving insulin were treated with growth hormone for five to eight days no rise in the insulin activity of the plasma was observed unless the dose of insulin was increased during the period of treatment with growth hormone. We conclude from these results that in the intact cat, treatment with growth hormone leads to a rise in the insulin content of the blood, and that this results from a greater rate of insulin secretion by the pancreatic islets rather than from a reduction of the rate of insulin utilization or destruction.

In the intact rat, in clear contrast to the results obtained in the cat, growth hormone treatment was without effect on the insulin activity of the plasma even though the hormone promoted significant extra growth under these conditions. This suggest that treatment with growth hormone does not alter the level of insulin in the blood of the intact rat. This conclusion does not necessarily mean that growth hormone is without effect on insulin secretion in the rat since the blood level of insulin is influenced both by the rate of insulin secretion and that of insulin utilization. Growth hormone might influence both processes without inducing any change in the blood level of insulin. These results are not at variance with those of Anderson and Long[4] and Scott and Engel,[10] which suggested that growth hormone might not promote the secretion of insulin by the pancreatic islets of the rat.

In man plasma insulin activity was found to be significantly enhanced in many cases of acromegaly[15] and this supports the possibility that in man too, growth hormone may promote the secretion of insulin by the pancreatic islets.

The evidence at present available suggests that growth hormone may promote the secretion of insulin by the pancreatic islets of the cat, dog and man but not by those of the rat Since growth hormone is diabetogenic in the cat, dog and man but not in the rat[1] it is possible that its action in stimulating insulin secretion in the cat, dog and man may be brought about indirectly through hyperglycemia, either latent or manifest. It is however necessary

to emphasize that none of the evidence at present available to support the view that growth hormone promotes the secretion of insulin by the pancreas, is conclusive.

THE INSULIN-LIKE ACTION OF GROWTH HORMONE

Under certain conditions pituitary growth hormone can cause a fall of blood sugar level, increase the uptake of glucose by certain tissues *in vitro* and accelerate the rate of entry of glucose into the eye from the blood, all actions of the hormone which resemble those of insulin under these conditions. The action of growth hormone in one or more of these respects will be referred to as the insulin-like action of growth hormone. Such actions are summarized in Tables I and II.

Growth hormone is not the only pituitary hormone with hypo-

TABLE I

IN VIVO INSULIN-LIKE ACTIONS OF GROWTH HORMONE IN ACUTE EXPERIMENTS

Hypoglycaemic Action in			
Rat	(Normal and Adrenalectomized)	Milman and Russell	(16)
	(Hypophysectomised-Eviscerated)	Park *et al.*	(8)
Mouse	(Normal)	Westermeyer and Raben	(17)
Dog	(Depancreatised)	Kurtz *et al.*	(18)
		Best and Sirek	(19)
Rabbit	(Normal)	Bulbrook and Ottaway	(20)
Man*	(Islet Cell Tumour)	Conn	(21)
No Hypoglycaemic Action in			
Dog	(Normal)	Foa *et al.*	(22)
Cat	(Normal)	Randle	(23)
Man	(Normal)	Carballeira *et al.*	(24)
	(Islet Cell Tumour)	Black *et al*	(25)
Rat	(Alloxan-Diabetic and Alloxan-Diabetic Adrenalectomised)	Milman and Russell	(16)
Accelerates Rate of Entry of Glucose into Eye in·			
Rabbit*	(Normal)	Ross	(26)

* Crude Growth Hormone preparations used

TABLE II

IN VITRO INSULIN LIKE ACTION OF GROWTH HORMONE

Stimulates Uptake of Glucose by:		
Diaphragm from:		
Normal rat (Stadie Buffer)	Ottaway	(2)
	Randle and Whitney	(27)
Insulin Treated Alloxan-Diabetic Rat	Ottaway	(2)
Hypophysectomised Rat (Krebs Henseleit Buffer or Geys Buffer)	Park *et al.*	(8)
	Randle	(23)
Mammary Gland Slices from:		
Lactating Rat	Folley *et al.*	(28)
Does Not Stimulate Uptake of Glucose by.		
Diaphragm from:		
Normal Rat (Krebs Henseleit or Geys Buffer)	Park *et al*	(8)
	Randle and Whitney	(27)
Alloxan-Diabetic Rat	Ottaway	(2)
Enhances Action of Insulin on Uptake of Glucose by:		
Diaphragm from Normal Rat	Randle	(29)

glycemic activity since corticotropin can induce a fall of blood sugar level in intact mice[18] and preparations of prolactin can cause a fall of blood sugar level in the intact dog.[29] The question arises whether the insulin-like action of growth hormone is due to contamination with corticotropin or prolactin, or whether the insulin-like action of all three pituitary preparations is to be attributed to a common contaminant, possibly an unidentified pituitary principle.

The ability of growth hormone to stimulate the uptake of glucose *in vitro* by the isolated rat diaphragm is an insulin-like action of the hormone not possessed either by corticotropin or prolactin. Thus growth hormone added *in vitro* markedly increased the uptake of glucose by diaphragms from hypophysectomized rats,[8, 24] whereas both prolactin and a corticotropin preparation (an oxycellulose concentrate of an acid acetone extract of pig pituitaries) were without influence *in vitro* upon the uptake of glucose by diaphragms from hypophysectomized rats (Fig. 1). The

insulin-like of growth hormone in this respect is not a property of corticotropin or prolactin.

Distinction between the hypoglycemic action of prolactin and growth hormone is provided by the influence of these hormones on the blood sugar level of intact and depancreatized dogs. In the intact dog, prolactin causes a fall of blood sugar level,[30] whereas growth hormone induces a rise (b); in the depancreatized dog pro-

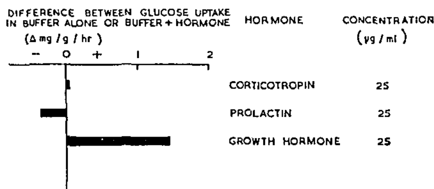


FIG 1 Influence of growth hormone, corticotropin and prolactin upon the uptake of glucose by the isolated rat diaphragm *in vitro*

lactin evokes a rise of blood sugar level[30] whereas growth hormone promotes a fall.[18, 19] Furthermore the observations of Park *et al.*[8] suggest strongly that the insulin-like action of growth hormone preparations is indeed a property of the hormone itself, for they found no dissociation between the growth promoting and insulin-like activities of different preparations of the hormone subjected to various inactivating or partially inactivating procedures.

The mechanism of the insulin-like action of growth hormone is obscure. The insulin-like action of growth hormone is not due to the release of insulin from the pancreatic islets under the influence of the hormone, since growth hormone is hypoglycemic in depancreatized dogs[18, 19] and eviscerated-hypophysectomized rats,[8] and furthermore the hormone *in vitro* has an insulin-like action on the uptake of glucose by the isolated rat diaphragm[2, 8, 27] and by mammary gland slices from lactating rats.[28] Nevertheless the

insulin-like action of growth hormone appears to depend upon the availability of insulin in the blood or tissues. Thus growth hormone is not hypoglycemic in the alloxan-diabetic or alloxan-diabetic adrenalectomized rat (in contrast to the intact or adrenalectomized rat,[16] nor is it hypoglycemic in the depancreatized dog deprived of insulin for 72 hours[19] (in contrast to the depancreatized dog deprived of insulin for shorter periods).[18,19] Furthermore Ottaway claims that although the uptake of glucose by diaphragms from normal rats is stimulated by the *in vitro* addition of growth hormone, growth hormone will only stimulate the uptake of glucose by diaphragms from alloxan-diabetic rats if the animals are injected with insulin before the diaphragm is excised. Further support for the view that the insulin-like action of growth hormone is dependent upon the availability of insulin is provided by the observation that growth hormone can enhance the action of insulin in increasing the uptake of glucose by the isolated diaphragm from normal rats[29] (Fig. 1). This enhancement of the action of insulin on the uptake of glucose by the isolated rat diaphragm is not however specific for growth hormone, since the effect has also been observed with preparations of corticotropin and prolactin (Fig. 2), but not with preparations of rat serum albumin. This action of corticotropin in enhancing *in vitro* the action of insulin on the uptake of glucose by the isolated rat diaphragm is not related to its ACTH activity, since preparations of corticotropin which have been inactivated with respect to adrenal ascorbic acid depletion (Sayers test) by treatment with periodate or peroxide were still capable of enhancing the action of insulin on the glucose uptake of rat diaphragm (Fig. 2).

Since the insulin-like action of growth hormone appears to depend upon the availability of insulin in blood or tissues, it has been suggested that growth hormone exerts this action by releasing insulin bound in blood or tissues. This view which has been advocated in particular by Ottaway[2] assumes that insulin can exist in blood or tissues in a bound form in which it is physiologically inert.

If growth hormone is able to release insulin from some inert combination in blood plasma, then the uptake of glucose by isolated normal rat diaphragms incubated in the presence of plasma

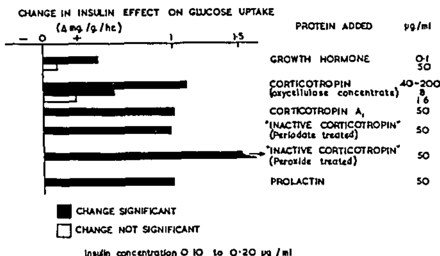


FIG. 2. Enhancement of the action of insulin upon the glucose uptake of the isolated rat diaphragm *in vitro* by growth hormone, corticotropin and prolactin

Samples of corticotropin prepared and donated by Dr. H. B. F. Dixon. Corticotropin was an oxycellulose concentrate of an acid acetone extract of pig pituitaries. Corticotropin A_1 was prepared from pig pituitaries by the method of Dixon and Stacke-Dunne.[40]

to which growth hormone has been added *in vitro* would be expected to be greater than that of diaphragms incubated in plasma without the addition of growth hormone. When tested in this way no evidence was obtained for an action of growth hormone in releasing insulin from normal human plasma or from the plasma of hypophysectomized rats.[14]

If the insulin-like action of growth hormone is due to the release of insulin bound in tissues in an inactive form, then tissues such as muscle must bind insulin at two sites, one at which insulin exerts its physiological action and one at which insulin exerts no action on the metabolism of the tissue. Such an idea could explain the action of growth hormone in increasing *in vitro* the uptake of glucose by the isolated rat diaphragm, and in enhancing the action of insulin on the uptake of glucose by the isolated rat diaphragm. On this basis growth hormone would exert these actions by displacing insulin from or by competing with insulin for binding by sites at which insulin exerts no effect on metabolism, without in-

terfering with the binding of insulin by sites at which it exerts an action on metabolism. There is at present no evidence for or against the view that growth hormone may exert its insulin-like action in this way.

The ability of corticotropin to enhance the action of insulin on the uptake of glucose by the isolated rat diaphragm does not appear to be due to competition between corticotropin and insulin for binding by sites at which insulin exerts no influence on metabolism. This has been tested by studying (a) the effect of corticotropin present throughout incubation on the uptake of glucose by normal rat diaphragms which have been allowed to bind insulin in a short preliminary exposure to insulin; (b) the influence of corticotropin bound to muscle in a short preliminary exposure to the hormone, on the action of insulin present throughout subsequent incubation, in increasing the uptake of glucose by normal rat diaphragms (c) the effect of corticotropin and insulin bound together to muscle during a short exposure to both hormones on the uptake of glucose by normal rat diaphragms incubated in the absence of both hormones. Under these conditions corticotropin does not enhance the action of insulin on the uptake of glucose by rat diaphragm, though in control experiments in which corticotropin and insulin were present throughout incubation, corticotropin enhanced the action of insulin in the usual way (Fig. 3).

The physiological significance of the insulin-like action of growth hormone is not clear. It is unlikely that endogenous growth hormone exerts an insulin like action, since hypophysectomy leads to an increase in the uptake of glucose by rat diaphragm[30] and not to a depression, such as would be expected if endogenous growth hormone were exerting an insulin-like action on the uptake of glucose by muscle. Furthermore animals are rendered resistant to the hypoglycemic action of growth hormone by prior treatment with the hormone[8, 16, 19] and intact rats are very much less sensitive to the hypoglycemic action of growth hormone than hypophysectomized rats.[8] The influence of growth hormone on the uptake of glucose by diaphragms from normal rats has been the subject of controversy. Park *et al.*[9] claimed that although growth hormone *in vitro* markedly stimulated the uptake of glucose by diaphragms from hypophysectomized rats, the hormone

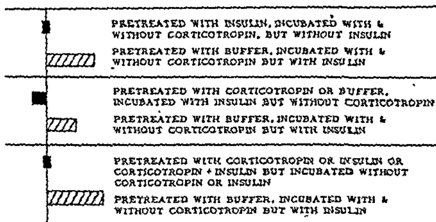


FIG. 3. Influence of corticotropin upon the action of insulin on the glucose uptake of the isolated rat diaphragm

Corticotropin—oxycellulose concentrate of an acid acetone extract of pig pituitaries

was without effect *in vitro* on the uptake of glucose by diaphragms from either normal rats, or, from hypophysectomized rats treated with growth hormone. Ottaway[2] on the other hand claims that growth hormone *in vitro* stimulates the uptake of glucose by diaphragms by normal rats. Randle and Whitney[27] find this discrepancy between the results of Park *et al.*[8] and of Ottaway[2] to be due to the difference between the buffer used for incubation by the two groups of workers In Geys' bicarbonate buffer (similar in composition to the Krebs-Henseleit buffer used by Park *et al.*) growth hormone *in vitro* was without effect on the uptake of glucose by diaphragms from normal rats. In the Stadie buffer (used by Ottaway) growth hormone *in vitro* had a small but significant effect in stimulating the uptake of glucose by diaphragms from normal rats The Stadie buffer by comparison with Geys or Krebs-Henseleit buffers lacks the ions potassium, calcium, sulfate and bicarbonate.

INHIBITION OF GLUCOSE UPTAKE BY GROWTH HORMONE

The investigations of Park and Krahl[31] and of Park *et al.*[8] have emphasized that the administration of growth hormone to nor-

terfering with the binding of insulin by sites at which it exerts an action on metabolism. There is at present no evidence for or against the view that growth hormone may exert its insulin-like action in this way.

The ability of corticotropin to enhance the action of insulin on the uptake of glucose by the isolated rat diaphragm does not appear to be due to competition between corticotropin and insulin for binding by sites at which insulin exerts no influence on metabolism. This has been tested by studying (a) the effect of corticotropin present throughout incubation on the uptake of glucose by normal rat diaphragms which have been allowed to bind insulin in a short preliminary exposure to insulin; (b) the influence of corticotropin bound to muscle in a short preliminary exposure to the hormone, on the action of insulin present throughout subsequent incubation, in increasing the uptake of glucose by normal rat diaphragms (c) the effect of corticotropin and insulin bound together to muscle during a short exposure to both hormones on the uptake of glucose by normal rat diaphragms incubated in the absence of both hormones. Under these conditions corticotropin does not enhance the action of insulin on the uptake of glucose by rat diaphragm, though in control experiments in which corticotropin and insulin were present throughout incubation, corticotropin enhanced the action of insulin in the usual way (Fig. 3).

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factor with inhibitory action *in vitro* on the uptake of glucose by rat diaphragm, whose presence in the serum was conditioned by the availability of growth hormone and adrenal steroid. Thus the inhibitor was lacking in the serum of alloxan-diabetic hypophysectomized rats (ADH rats) but was present in the serum of ADH rats treated with both growth hormone and cortisone. By contrast, the serum of ADH rats treated with either growth hormone

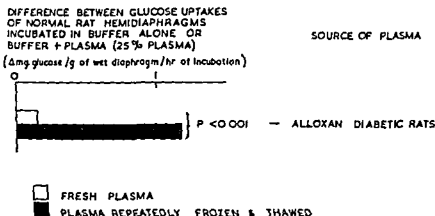


FIG 4. Influence of plasma from alloxan diabetic rats upon the glucose uptake of the isolated rat diaphragm *in vitro*.
Plasma frozen and thawed at least three times in solid carbon dioxide/acetone, and water at 37°C.

or cortisone was devoid of inhibitor. These observations, which have been confirmed in our laboratory by Dr J. E. Whitney suggest that an inhibitor may be produced *in vivo* from or under the influence of growth hormone and adrenal steroid which is responsible for the depression of glucose uptake by muscle produced *in vivo* by growth hormone.

In further studies of the serum inhibitor Bornstein[33] obtained evidence that the inhibitor might be a beta lipoprotein. Thus the inhibitor was located in the fraction of serum proteins containing beta lipoproteins and was inactivated by repeated freezing and thawing. Observations made in this laboratory would be consistent with the view that this inhibitor is a lipoprotein. Thus although fresh plasma from alloxan diabetic rats did not stimulate the up-

mal or hypophysectomized rats leads to a profound depression of the glucose uptake of muscle as measured by the uptake of glucose *in vitro* by the excised diaphragm. Krahl and Park[30] found too that the uptake of glucose *in vitro* by diaphragms from hypophysectomized rats was greater than that of diaphragms from normal rats. Park *et al.*[8] reported that the uptake of glucose by diaphragms from hypophysectomized rats could be reduced to normal levels by a single injection of growth hormone before removal of the diaphragm. This action of growth hormone was delayed in its appearance, and followed a period during which treatment with the hormone had led to an increase in the uptake of glucose by the diaphragm (a manifestation of the insulin-like action of growth hormone). The development of this inhibitory action of growth hormone on the uptake of glucose by muscle appeared to be dependent upon the availability of adrenal steroid, since this effect was seen in hypophysectomized-adrenalectomized rats only when a small dose of adrenal steroid (itself without effect) was given concurrently with growth hormone.[8]

The nature of the inhibitory action of growth hormone on the uptake of glucose by muscle has been the subject of controversy. Thus Park *et al.*[8] observed that although *in vivo* treatment with growth hormone led to a depressed uptake of glucose by the isolated diaphragm of normal or hypophysectomized rats, the addition of growth hormone *in vitro* did not depress the uptake of glucose by diaphragms from normal or hypophysectomized rats. Bulbrook and Ottaway[32] on the other hand claim that the uptake of glucose by diaphragms from normal rats is depressed by the addition of growth hormone to the suspending fluid. In studies in this laboratory of the influence *in vitro* of many preparations of growth hormone upon the uptake of glucose by isolated diaphragms from normal or hypophysectomized rats inhibition of glucose uptake has not been observed.[14, 23, 25]

Park *et al.*[8] suggested on the basis of their observations that the depression of glucose uptake by muscle induced *in vivo* by growth hormone might depend upon the transformation of growth hormone *in vivo* to another molecule. Subsequently Park and Bornstein[9] claimed that the serum of alloxan diabetic rats contained a

in the serum of depancreatized cats deprived of insulin, treated with insulin, or treated with insulin and growth hormone (Fig. 5).

This serum inhibitor whose formation is dependent upon the availability of both growth hormone and adrenal steroid does not represent the only mechanism by which growth hormone inhibits glucose utilization *in vivo*. Thus Houssay[34] has shown that growth hormone is diabetogenic in the adrenalectomized-partially depancreatized dog maintained with sodium chloride, and de Bodo[35] claims that growth hormone will induce resistance to the hypoglycemic action of insulin in the hypophysectomized-adrenalectomized dog receiving sodium chloride or desoxycorticosterone as supportive therapy. Thus it seems probable that growth hormone has an influence in depressing glucose utilization and antagonizing insulin action which is independent of the adrenals. This adrenal independent action of growth hormone may be directed at preventing the conversion of glucose to fat, since Bowen and Perry[36] claim that lipogenesis from glucose or acetate is inhibited in the livers of adrenalectomized rats treated with growth hormone.

GENERAL CONCLUSIONS

The administration of growth hormone or growth promoting pituitary extracts to intact rats leads to changes in carcass composition such as to suggest that growth hormone depresses the utilizaion of carbohydrate, promotes the catabolism of fat and the deposition of new protein.[1, 37, 38] Although carcass analyses are lacking in the cat and dog, metabolic studies suggest that in these species too, growth hormone depresses the utilization of glucose, promotes the catabolism of fat and the deposition of new protein.[1] In the cat and dog growth hormone may still promote nitrogen retention, despite the fact that the utilization of glucose is so severely depressed as to lead to diabetes.[1] These principal metabolic actions of growth hormone are summarized in Table III.

Thus the growth hormone promotes nitrogen retention under conditions where glucose utilization is depressed and where the energy for protein synthesis is presumably provided by the oxidation of fat. The depression of glucose utilization in muscle under the influence of growth hormone is dependent upon the availabil-

take of glucose by the isolated rat diaphragm, the same plasma after repeated freezing and thawing did stimulate glucose uptake (Fig. 4). This effect of freezing and thawing in inactivating the serum inhibitor has been used as a crude test for the presence of

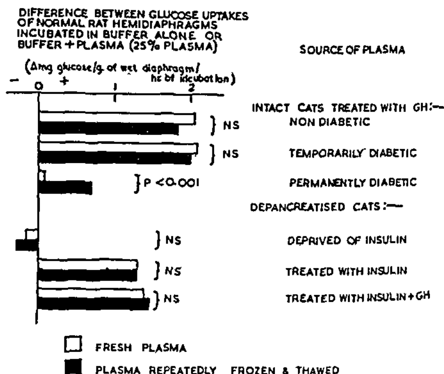


FIG. 5. Influence of plasma from diabetic cats upon the glucose uptake of the isolated rat diaphragm *in vitro*.

Plasma frozen and thawed at least three times in solid carbon dioxide/acetone, and water at 37°C.

Animals taken from food and injected with growth hormone (and insulin where this was given) 80 minutes before blood was collected.

the inhibitor in samples of plasma from other types of diabetic animals. With this test the inhibitor could be identified in the serum of cats with permanent diabetes induced with growth hormone (metahypophyseal diabetes). The inhibitor could not be identified in the serum of cats with temporary diabetes induced by treatment with growth hormone (idiohypophyseal diabetes) nor

growth in the absence of growth hormone, it seems probable that the influence of growth hormone on nitrogen retention and growth may be brought about by insulin. Since under the influence of growth hormone, insulin exerts this effect on protein anabolism under conditions where the utilization of glucose is depressed, this action of insulin on protein anabolism is presumably not mediated through its action on carbohydrate metabolism, and may represent a direct action of the hormone on protein synthesis.

Sinex, McMullen and Hastings[42] in their studies of the influence of insulin on the incorporation of labeled alanine into the proteins of the rat diaphragm *in vitro*, observed that the incorporation of labeled alanine was inhibited by glucose. This inhibitory action of glucose was most marked in the presence of insulin which, in the absence of glucose, accelerated the incorporation of labeled alanine into protein. Krahl[43] on the other hand observed that insulin or glucose could accelerate the incorporation of labeled glycine into the proteins of the isolated rat diaphragm *in vitro*. When both insulin and glucose were present, the rate of uptake of glycine was the same as with insulin or glucose alone, presumably because either factor could promote the maximal rate of incorporation of glycine. Russell[44] evaluated the influence of growth hormone on protein anabolism by measuring the rate of formation of urea from amino acids in the eviscerated nephrectomized rat. Russell observed that the protein anabolic action of growth hormone was most marked when fat or its metabolites were fed or injected. When glucose was injected or a high carbohydrate diet fed, protein anabolism was stimulated, but under these conditions growth hormone itself exerted no effect on protein anabolism.

Thus it would appear that growth hormone enables insulin to exert a direct influence on protein synthesis under conditions where the action of insulin on carbohydrate metabolism is restrained and fat catabolism is enhanced. The mechanism by which growth hormone emphasizes the protein anabolic influence of insulin and initiates additional protein synthesis is not clear. There is evidence, as has already been indicated, that growth hormone may promote the release of insulin from the pancreatic islets or

TABLE III

PRINCIPAL METABOLIC CHANGES INDUCED BY GROWTH HORMONE

Metabolic Change	*Brought About By*	*Co-Factor*	*Reference*
Carbohydrate Metabolism:			
Depressed utilisation of glucose by muscle	Growth Hormone (? lipoprotein)	Adrenal Steroid	(8, 9, 31, 33)
Depressed conversion of glucose to fat	Growth Hormone	—	(36, 39)
Enhanced production of ribose and glucosamine	?	?	
Fat Metabolism:			
Fat mobilisation	? Growth Hormone ? Adipokinin	Adrenal Steroid	(45)
Fat oxidation stimulated	Growth Hormone	?	(1, 37, 38, 46)
Fat synthesis depressed	Growth Hormone	—	(36, 39, 47)
Protein Metabolism:			
Protein synthesis enhanced	? Insulin	Growth Hormone (? lipoprotein)	(40, 41)
? Protein breakdown reduced	?	?	(44, 48)

ity of adrenal steroid, and may be brought about by a lipoprotein inhibitor formed from or under the influence of growth hormone and adrenal steroid, though the exact significance of this inhibitor and its relationship to the diabetogenic action of growth hormone have yet to be defined. The inhibition of lipogenesis from glucose brought about by growth hormone[36, 39] is not dependent upon adrenal steroid[36] and is presumably a direct effect of growth hormone and not dependent upon its *in vivo* transformation to an inhibitor of the type described by Park and Bornstein.[33]

The investigations of Lukens and McCann[40] and of Salter and Best[41] suggest that *growth hormone promotes nitrogen retention* and growth only when insulin is available. Since their studies showed also that insulin can promote nitrogen retention and

interesting possibilities would be the formation of ribose from *glucose by the glucose-6-phosphate oxidative pathway,* and the formation of glucosamine from glucose, since these substances would be necessary for the deposition of new tissue.

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tissues under conditions where the action of insulin on carbohydrate metabolism is sufficiently restrained for there to be no danger of hypoglycemia and damage to the central nervous system. That such an action of growth hormone in stimulating insulin secretion could lead to nitrogen retention was clearly demonstrated in the cat by Milman, de Moor and Lukens.[11] However they showed also that growth hormone could promote nitrogen retention in the cat under conditions where additional pancreatic insulin was not available i.e. in the depancreatized cat receiving a constant amount of insulin by injection. Furthermore as has already been indicated, evidence is lacking for the secretion of extra insulin in the rat growing under the influence of growth hormone. The experiments of Sinex, McMullen and Hastings already referred to, suggest the possibility that growth hormone may emphasize the action of insulin on protein anabolism by restraining carbohydrate utilization, but their experiments are open to the technical objection that the labeled alanine may have been diluted by transamination with unlabeled pyruvate derived from glucose. Thus all that can be said at the present time is that protein anabolism is stimulated by growth hormone in the presence of insulin under conditions where carbohydrate utilization is depressed and fat catabolism is enhanced, and the means by which increased protein synthesis is brought about remains obscure.

Since growth hormone depresses the utilization of glucose by muscle and the conversion of glucose to fat, it is scarcely surprising that growth hormone is diabetogenic in the cat and dog. Indeed it is the more surprising that growth hormone is not diabetogenic in the rat, for the hormone in this species too depresses the utilization of glucose by muscle[31] and lipogenesis from glucose.[39] Furthermore, as already indicated, the lack of a diabetogenic action of growth hormone in this species does not appear to be due to the secretion of additional insulin by the pancreatic islets under the influence of growth hormone. Since the rat does not develop diabetes when treated with growth hormone, there must in this species be some pathway, in some tissue other than muscle, whose capacity to dispose of glucose is not impaired by growth hormone treatment. This pathway has yet to be identified but

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DISCUSSION

DR. SAMUELS: I am glad that Dr. Randle brought in the possible role of the glucose shunt because, since this has received little discussion, I am anxious to hear Dr. Stetten say something about this subject.

DR. STETTEN: I don't have a great deal to say. As you are perhaps all aware, various attempts in various laboratories including my own have been made to evaluate the abundance of glucose entering the phosphogluconate oxidative pathway, as contrasted with that entering the traditional Embden-Meyerhoff glycogenolytic pathway. These studies have moved slowly, largely because the methods in use have required several assumptions. I know of no studies in which growth hormone as such has been applied in attempts made to evaluate changes in the abundance of these pathways. Perhaps Dr. Randle does know of these. There are two findings, however, that may conceivably be related to the problem. One is a recent observation from Brookhaven by Gibbs, I believe, in which he determined the specific activity of carbon dioxide produced *in vitro* from 6 and 1 labeled glucose, by various portions of the plant. I believe his findings may be summarized, by stating that in those portions of the plant which are most actively growing, one finds an abundance of the phosphogluconate oxidative pathway, in contrast to the Embden-Meyerhoff pathway. In those parts of the plant where growth is less active the reverse is true. Very recently my colleague Dr. Bloom in conjunction with Dr. Harry Eagle, has carried out tissue culture studies using the so called Heloff strain, and a connective tissue strain. They find that in both of these tissue culture preparations, far more CO_2 is derived from carbon 1 of glucose than from carbon

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DR. HOUSSAY: Really I cannot answer your question. We have seen both actions. That is all that we can say up to now.

DR. GORDON: The question that Dr. Astwood has asked may be pertinent to certain aspects of juvenile diabetes. We frequently see children who are in the process of developing their disease. It appears to be characterized by a preliminary period of gradual loss of glucose tolerance, until they become "decompensated." A diabetogenic factor appears to be operating. This process may be reversible up to a certain point. This seems to duplicate every detail of Dr. Young's original experiments. If, one starves such a child for several days he can be temporarily recompensated, so to speak. Intensive insulin administration may do the same thing. I regard this as the state of hydropic degeneration of the Islet cells, presumably a reversible process. I have never seen one of these children who did not in the space of two or three months or perhaps a year, progress to permanent diabetes. Presumably a diabetogenic factor continues to operate, and eventually makes these children permanently diabetic.

DR. HOUSSAY: The answer to the problem that you have presented is not easily obtained. In the pancreas of the juvenile diabetic Dr. Ransome has shown that there is a striking decrease of insulin. In the pancreas of the adult with diabetes mellitus, the condition is variable. Sometimes there is much insulin That is a difference that I cannot explain. We have observed hydropic degeneration (glycogen infiltration), in cats, turtles and dogs. We have studied turtles very much. There is a great difference between rats and dogs and cats. In cats and dogs it is very easy to obtain diabetes. In rats it is extremely difficult.

Until now I have not been able to cure diabetes in partially depancreatized dogs. Rats do not get diabetes easily. It is possible to cure diabetes in rats, and we have used, for instance, insulin and thiouracil. With insulin alone it is sometimes possible to cure diabetes in cats in the first stages. That has been demonstrated by Lukens and other people. By giving insulin and estrogen we have cured two thirds of diabetic rats—none with insulin alone. Thiou-

6, the ratio in one experiment, as I recall it, being of the order of one hundred to one. This would indicate that glycolysis followed by Krebs cycle oxidation is not abundant in rapidly growing tissue cultures. Ribose, levulose and related compounds are formed by this process. One must keep in mind however that the Embden-Meyerhoff sequence may be operating but the beta carbon of pyruvate thus produced is not available for carbon dioxide production, since, during growth, these precious 3 carbon fragments are used for the synthesis of amino acid skeletons, sugars, and fatty acids. We have used muscle and liver from diabetic animals and normal animals in recent studies. In muscle it has been our finding, and I think the finding of almost anyone who has studied in this field, that there is no evidence of the occurrence of an intact phosphogluconate pathway. However, in liver, according to various estimates, anywhere from 15 to 60% of the glucose normally utilized by normal liver slices is catabolized over the phosphogluconate pathway. When the liver is derived from a diabetic animal total glucose utilization is markedly suppressed and both pathways are suppressed. It would appear that the glycolytic pathway is more extensively inhibited in the diabetic liver slice than is the phosphogluconate oxidation pathway

DR. ASTWOOD: I would like to ask Dr. Houssay a question. As you have so nicely shown, if one removes the pituitary gland from a depancreatized animal the state of diabetes mellitus is relieved. Administration of pituitary extracts, including growth hormone preparations, restores the diabetes. If one injects the normal cat or dog with crude pituitary extracts or growth hormone fractions, one likewise induces a state of diabetes which, as Young has shown, may become permanent. There is some evidence that suggests the diabetogenic effect is somehow mediated by some action on alpha or beta cells or their secretory products. I want to know your opinions, Sir, on whether the substances concerned are the same in the two cases. In the one case one is restoring diabetes in the depancreatectomized hypophysectomized animal, and in the other case one is damaging the pancreas of the normal animal. Are these the same effects?

and the Gomori stain showed abundance of granules. We did not do insulin assays however.

Dr. Houssay's remarks and Dr. Astwood's question, prompts me to ask another one in the same line. In pancreatic diabetes of animals, which is an expression of lack of insulin in the presence of an intact pituitary, the animal is normally insulin-sensitive. When the hypophysis is removed, the animal becomes more insulin-sensitive. When one gives back crude pituitary extract or growth hormone the diabetes may return to its previous state. The hyperglycemia and the glycosuria will be the same, as will the ketosis, but added to that will be an insulin resistance not previously found. How can we explain this phenomenon?

We must bring a little more precision into the term insulin resistance or insulin antagonism, even as used by Dr. Randle in his *in vitro* work. He used rat diaphragms and observed glucose uptake as a measure of insulin activity. He is measuring the disappearance of glucose from the medium into the tissues. This is the consequence of a sequence of reactions. First, the entrance of glucose into the cell, then the hexokinase reaction, then all the other reactions of glucose disposal. The totality of this we measure as glucose uptake. If insulin acts (as we think it does) on the sugar transfer reaction, then glucose uptake is not really a measure just of insulin action. A better measure of insulin action might be the use of a material which is not further metabolized. If you measure this in the eviscerate preparation or in the diaphragm, then cortisone and growth hormone which may antagonize the rate of glucose uptake do not inhibit the rate of the transfer reaction. If you put this information together with some of the lipoprotein studies of Bornstein and Krahl(11) in which a hexokinase extract was inhibited, but insulin reversal was only occasionally obtained, it is possible to postulate that cortisone or G H. inhibit the hexokinase reaction (and thus inhibit glucose uptake) but they do not oppose insulin at its specific site of action I think that *in vitro*, if one wants to study materials thought to enhance or inhibit the action of insulin it might be better to

[11] KRAHL, M E AND BORNSTEIN, J. *Nature*, 173:949, 1954

racil decreased the intensity of the diabetes during treatment. The moment we stop treatment, the diabetes relapses. In the different kinds of diabetes, there have been reports of differences between species. In dogs and in cats one sees degeneration (hydropic), degranulation, and after some time progressive decrease of beta cells. In the turtle there is infiltration with glycogen, and hyperplasia of the Islets. It is impossible to produce degeneration or diabetes. I have the impression that so called glycogen infiltration is related in a major way to hyperglycemia. Glycogen infiltration, for unknown reasons, is harmful for the pancreas and probably for other tissues, e.g., the kidney. It can be said of the kidney when there is glycogen infiltration for some time, there is soon atrophy of the cells.

About the permanent diabetes, we have obtained permanent diabetes before Young in 1932, but we have obtained that only in animals with partial pancreatectomy and total pancreatectomy. We have noted toxic symptoms such as DeBodo has described. They had vomiting, anorexia, and it was necessary to interrupt the injections. Dr. Young's extract was in some way different. Dr. Young has studied very well these types of diabetes. He is not the first but he is the man who has studied the problem to the best advantage. What is the reason for the temporary diabetes becoming permanent diabetes? That is a problem unsolved until now. In the stage of temporary diabetes it is very difficult by inspection to see abnormalities in the Islets of Langerhans. In doing grafts of pancreas to other animals, it is possible to see that there is some difference in the secretion of insulin. During temporary diabetes there is diminution but not suppression of insulin. When there is permanent diabetes there is total suppression of insulin.

DR. LEVINE: Regarding Dr Gordon's question about the youngster with diabetes. Perhaps there the diabetogenic factor is acting on a low pancreatic reserve. This seems to be different from the situation in acromegaly with diabetes. At least in two cases which we have observed over a long time, both with severe diabetes, which persisted even after the acromegaly itself had become quiescent, at autopsy the Islet cells were if anything more abundant

served that puppies when compared with adult dogs were resistant to the diabetogenic effects of growth hormone and of partial pancreatectomy. They may be resistant because the pancreas is able to supply insulin more readily than in adults, or alternatively the demands for insulin in the young growing animals may be less than that of the adult animal under the influence of growth hormone.

DR. JOSLIN: May I supplement and concur with what Dr. Gordon said. We, too, have never cured a diabetic. Repeatedly though we have seen exactly what he described about the "almost disappearance," not disappearance, but the decrease of the severity of the diabetes in the child. I would carry the idea a little bit further. It fits in with our 63 Quarter Century Victory Medal diabetics, who after 25 years, when examined by expert ophthalmologists, show no alteration of the eyes; by expert roentgenologists, no trouble with the arteries in the ankles, legs and pelvis, so important in pregnancy, or in the aorta; no trouble with the kidneys, and no elevation of the blood pressure. If you study the histories of these 63 cases, you will find that they were treated meticulously and with very low calorie diets. Almost all of them practically starved in their first years of diabetes. That is a very striking factor about those cases. It is a great sorrow to me now that with all of our children, we have 3,700 of them, 700 dead and about 3,000 alive, that we do not find in our own group more Victory Medal cases. I think one of the reasons is that we do not treat them with as great care and undernutrition as we did formerly. Maranon, years and years ago, spoke of the long duration of the incipiency of diabetes. Umber, long before he died, talked about the advantage of over-insulinization, and Brush emphasized it in a series of cases. Our cases who get the medals were treated with great severity at the start. May I illustrate this by the Ilsley boy who came to me on December 7 of last year. He played end at 16 years of age on the Everett football team. He was 6′ 1″ tall. He played all through the fall in perfect condition. He even played on Thanksgiving day, but a week thereafter, when quite inactive, yet probably eating as he did while playing football, he suddenly developed a 450 mg. blood sugar and 4.8% glycosuria. In 10 days, with rest in bed to

use the first reaction only. I wonder if you agree with that Dr. Randle?

DR. RANDLE: I agree with you in part, Dr. Levine. The essence of Park's view of the process of transfer of glucose into the cell as I recall it is that transfer, and not the hexokinase reaction, is the rate limiting step in the uptake of glucose by diaphragm. Once the glucose gets into the cell its disposal is so rapid that none accumulates except under very special conditions. (Park, C. R.: In the *Hypophyseal Growth Hormone—Nature and Actions*. New York, McGraw Hill, 1955.) Can you be sure that the condition of transfer of glucose is the same as that for non-metabolizable sugars?

DR. LEVINE: No I cannot be sure that the transport mechanism is the same except that it is reasonable to take the simplest hypothesis at the moment, because of the specificity of the varying sugars involved, the transfer system is probably the same, and influenced by the same hormone.

DR. RANDLE: I would like to raise a problem in relation to human diabetes. The results of Dr. Priscilla White's studies suggested very strongly that in juvenile diabetics, the appearance of the diabetes was heralded in many instances by a period of abnormally rapid growth so that these juvenile diabetics were, for their age, overheight. This suggests the possibility that abnormally rapid growth, whether initiated by hypersecretion of growth hormone or not, might be a factor which can precipitate diabetes. On the other hand if one compares pituitary gigantism with acromegaly, diabetes is relatively common in acromegaly but is not commonly seen in pituitary giants. The latter observation would fit with the experimental observations of Young which showed that although growth hormone is diabetogenic in the adult cat and dog it is without diabetogenic effect in the kitten and puppy.

The question thus arises as to the significance, at any rate in relation to growth hormone, of the abnormal growth which is sometimes seen in juvenile diabetics. There is one further point, and that is in relation to the experiments of Langfeldt who ob-

of 8, 10 or 12 years show an excessively high incidence of abnormal metabolism, with either typical changes in the glucose tolerance test or actual diabetic symptoms. Many of them tend to be short rather than to have excessive height.

DR. BEST: The papers given by Dr. Randle and Dr. Houssay this morning raise so many questions that I find myself inhibited by numbers. I suppose we will have to wait until the publication of the proceedings of this conference to secure all the details in which we are interested. Dr. Campbell in our laboratory has been studying in Houssay dogs recently very much the same points that Dr. Scow and Dr. Houssay have been investigating in the rats deprived of both pancreas and pituitary. Insulin alone gives a nice nitrogen retention. This confirms the work that Dr. Lukens had done in cats. Some preliminary work has been done by Dr. Salter with alloxan diabetic hypophysectomized rats. The growth effect of insulin that Dr. Randle mentioned is easily seen in hypophysectomized animals, but is not seen on a restricted diet. When carbohydrate alone with nothing else added is given, a very marked increase in nitrogen retention is secured with insulin. Dr. Gordon's question I found very stimulating but I wondered why he said that the picture in children was the same as in the dogs made diabetic by pituitary extracts. What evidence is there that this is due to an excess of pituitary secretion? Without estimation of the various substances which may work antagonistically to insulin, you will agree that one cannot tell how many of these substances may be acting in the diabetic child. The situation would be better if we could measure the concentration of growth hormone in their blood. I suppose clinically you are able to secure an approximation of the level of thyroid and adrenal substances. I would like to hear more details from Dr. Gordon, who knows the experimental field, as well as the clinical.

DR. GORDON: I think you are quite right, Dr. Best, in challenging such a statement as I made. However, I refer to it only as a tentative hypothesis formulated in my mind because it seems that the conditions under which diabetes develops in children, show a lot of superficial parallelism to the development of experimental dia-

save calories, with 30 units of insulin and a low diet he was discharged. On December 24 he was sugar free, with 38 units. I am doing everything I can to prevent that boy from growing another inch or gaining weight, because I am hoping to protect his carbohydrate metabolism. I know that the children we starved were the ones who have done the best. I am wondering whether with the obvious activity of the growth hormone in children, we are not throwing fuel on the flame if we overfeed them. In a way this boy was exposed to the development of acute obesity.

I am sure that if you saw these children as we do that you would be impressed with their excess height. When I see children in diabetic families who are overweight and overheight, I try my best to protect them from acquiring diabetes by warning their parents not to let them overeat.

DR. ROOT: The difference in the incidence of diabetes in pituitary giants who attained excessive height before the end of the growth period, and the acromegalic should be emphasized. We went over a series of acromagalics some years ago. Among the 150 patients, some 29 had developed diabetes. These patients have been followed up for a number of years. The striking fact was that the acromegalics who developed diabetes were the ones with a known family history for diabetes. It appeared that hyperpituitarism did not seem to be very effective even after eight or 10 years, unless there was a definite hereditary background. In connection with excessive height of diabetic children, it may be remembered that in 417 children whose examination took place within three months of the onset of diabetes, in 85% of the group the Baldwin and Wood standard of height for age had been exceeded. Actually, in another study, similar results were found even with the use of other standards for height. Excessive bone growth was also shown. Nevertheless, the fact remains that there is a group in whom growth has not been excessive, but who nevertheless developed diabetes.

Priscilla White has recently given us some figures on young diabetic mothers and their children. These diabetic mothers have been severe diabetics, most of whom have developed diabetes during their childhood. Their babies by the time they reach the age

pressed by a watch mechanism which will deliver the daily dose of insulin continuously over the 24 hours. I'd like to see this tried clinically but I realize that there may be insurmountable difficulties. Some sort of a none irritating silicone catheter that could be inserted in some area where the insulin would not be walled off might give us a better type of treatment than has been obtained previously. Of course this would be a long experiment extending over 15 or 20 years, but if we could overcome the mechanical difficulties of administration I think that such a study in a certain number of patients over a long period of time would be well repaid.

DR. JOSLIN: Dr. Root will supplement me if I do not state things correctly. Dr. Priscilla White has ministered to many babies born at the Boston Lying-in Hospital. Recently at the Peter Bent Brigham Hospital, which is adjacent, one kidney of a healthy twin was grafted into the body of the twin who had diseased kidneys. Dr. White and others at the Brigham Hospital and at the Boston Lying-in Hospital have been on watch for one of our diabetic mothers whose baby soon after birth might die suddenly. One such mother gave permission that if her baby died the baby's pancreas could be grafted into her own body. This was done within 23 minutes after birth. The pancreas graft is still intact. It is now about three months. By this means one furnishes the diabetic mother with a possible source of insulin. What the result will be we do not know. So far there is nothing striking.

DR. STETTEN: I have two questions. What is the present status of some older observations which I recall, that the continuous phloridzination of the appropriate animal prevents or markedly retards the development of permanent metahypophyseal diabetes? The second question: is it possible that the glycogen accumulation observed in the beta cells in animals receiving growth hormone is a consequence, not a cause, of the defect in the cell? I say this in reference to a comment made by Dr. Lardy yesterday with which I am in complete accord, that glycogen is in a sense a cul-de-sac; that glycogen is deposited in tissues when the tissues have nothing better to do with the glucose that is supplied to them. Moreover,

betes in "Young dogs." They seem to go through the stage that Dr. Randle has referred to as the idiohypophyseal stage, which appears to be reversible to a degree, as indicated by the things that both Dr. Joslin and I referred to. Additional information, either confirmatory of contradictory, could be obtained if someone would measure the amount of insulin circulating in the blood of these children, using Dr. Randle's technique. This stage of idiohypophyseal diabetes later changes to the stage of "metahypophyseal diabetes." This is not reversible. This concept is advanced only as a working hypothesis.

DR. BEST: I think that Dr. Gordon's suggestion is a most valuable one but it does require a lot of proof. I'm not just sure that you can compare the young dog, which is most difficult to make diabetic according to the work of Dr. Young and his colleague, with the young human. The pup is difficult to make diabetic with growth hormone until it stops growing.

DR. RANDLE: That is certainly true. Young showed clearly that growth promoting pituitary extracts which were diabetogenic in the adult cat and dog did not elicit diabetes in the kitten or puppy (Young, F. G: *Brit. Med. J. 2*:897, 1941). I have recently had occasion to give purified growth hormone to kittens in amounts which would certainly elicit a diabetic response in the adult cat. No diabetes was seen in the kittens receiving growth hormone. That is partly why I raised the question of the difference between the pituitary giant and the acromegalic, with respect to incidence of diabetes. I think that Dr. Best has made a very important point. Growth hormone would not be expected to be diabetogenic in the growing child.

DR. BEST: Without diminishing the importance of these substances which are intensely diabetogenic it would seem to me that we never give insulin under completely physiological conditions to human subjects. There is never complete freedom from diabetes throughout the day. None of the delayed action insulins is perfect. We have devised an apparatus which may be used experimentally, but not clinically, in which a little 2 cc. capsule is com-

enough insulin, but in these animals, when we added growth hormone, there was markedly increased growth. I would like to know then if Dr. Best has done these experiments in hypophysectomized animals giving them food alone, and the same amount of food plus insulin.

DR. BEST: In the experiments which Dr. Salter has done with me the growth of the hypophysectomized animal injected with insulin is dependent upon an increase in caloric intake in the form of carbohydrate. The carbohydrate cannot apparently be replaced by fat. The growth hormone treated animals use labile fat from their own stores but for the growth of hypophysectomized animals with insulin alone there is usually not enough stored carbohydrate, and this has to be supplied. This growth effect of insulin in hypophysectomized animals has been seen only with protamine zinc insulin. When forced feeding is studied in animals with intact pancreas, the most suitable control is the depancreatized animal, force fed the same diet. This will reveal the role of insulin as a growth hormone. If hypophysectomized animals receive a certain food intake, and sugar is added to the diet of these receiving insulin, you can get a fine nitrogen retention in response to insulin. Dr. Salter has found that this is equivalent to that obtained with growth hormone under comparable conditions.

The glycogen infiltration of beta cells which Dr. Duff and Dr. Toreson pointed out, disappeared when insulin was given, and the blood sugar maintained at exactly the same height. It was not the level of the blood sugar that seemed important but the absence of insulin.

DR. HOUSSAY: I would like to add this one thought. It is much more accurate and simple to feed the animals by force feedings, as you avoid hypoglycemia and don't need to be there all the time to feed them. I think a force feeding experiment of this type should be done to confirm or deny these results.

DR. LEVINE: I should like to recall an old experiment of Ranson's that seems to support the view that it isn't food intake alone in

I assume that the beta cell placed in an environment rich in glucose uses the energy which it can derive from such glucose to make extra insulin, but if a beta cell cannot use glucose in this situation it makes glycogen simply because it has no other use for the glucose with which it is supplied.

DR. HOUSSAY: I remember when I visited Dr. Joslin in 1935 that he showed me a lot of young diabetic children with less growth than the average. I don't remember how many but there were 20 or so. Before the development of treatment with insulin there were many dwarfed children. That is very typical in diabetic animals. However, these cases were well treated but were still below normal size. I mention this to indicate that we have no evidence that growth hormone or any pituitary factor is the cause of diabetes in man. We are still unable to measure these hormones in man directly. Also there is evidence that in man the growth hormone obtained from other animals is usually not active. For instance we know that the growth hormone that we inject usually does not cause growth in man and in the Rhesus monkey. Also, it does not produce diabetes in the Rhesus. I remember that Dr. Knobel spoke to me about that, and I recommended that he do partial pancreatectomy. Still he was not able to produce diabetes. He has told me that he will try to use growth hormone prepared from the pituitaries of Rhesus monkeys that have been used in preparing Salk vaccine. I do not know what the results have been from this, but I expect positive results.

About the interesting problem of insulin and growth. I'm interested in the very important experiment of Dr. Best. When you inject insulin in animals, hypophysectomized or normal, they will eat much more. The question that I would like to ask Dr. Best is, did not the animals that you injected with insulin eat a great deal more, and was that not the reason for their growth? I would like to do this experiment with force feeding. We have done experiments with hypophysectomized animals who have received 11 grams of food and 3 units of insulin per day, and others who received food without insulin. Growth occured equally in each group. Completely the same. Three units of insulin was not enough to change the rate of growth. Perhaps we did not use

a very clear difference in nitrogen balance between the depancreatized animal, deprived of insulin which has a negative nitrogen balance, and the idiohypophyseal diabetic animal which has a positive nitrogen balance.

DR. KINSELL: Cortical steroids, as well as the "diabetogenic factor" associated with growth hormone are frequently referred to as being anti-insulin, i.e., as diminishing carbohydrate utilization. It seems to us that the total available evidence would suggest that both factors *increase the demand for insulin* instead of interfering with the action of insulin. A truly diabetogenic effect occurs only if the beta cells are unable to keep up with the demand. Obviously, the latter part of this hypothesis requires documentation.

One question I should like to ask Dr. Randle: do you have any blood insulin levels in early juvenile diabetics?

DR. RANDLE: I have no estimates of plasma insulin levels in juvenile diabetes.

I have a question which I should like to ask Dr. Stetten. What is the quantitative significance of the two main pathways of glucose utilization, the one the conversion of glucose to fat and the other the utilization of glucose by muscle. I think this is important, because there is evidence that growth hormone depresses glucose utilization in both respects, whereas insulin increases glucose utilization in both respects I think that the quantitative evaluation of these two pathways of glucose disposal may be important because the experiments of Salter and Best showed that in the hypophysectomised rat insulin causes nitrogen retention and fat deposition, whereas growth hormone causes nitrogen retention and loss of body fat (Salter and Best: *Brit. M. J.* 2:353, 1954).

DR. ROOT: Concerning the child who initially requires insulin but later has a remission, are there any determinations of insulin in such children during the early weeks or months and particularly during the period of remission in which the urine is sugar-free and the blood sugar normal? The general idea which we have had from Dr. Haste and Dr. Bornstein's data is that the childhood cases of diabetes become practically devoid of insulin either in the

the hypophysectomized animal that makes them grow, but that there must be some other factor. Ranson and Hetherington produced hypothalamic lesions and caused obesity on the basis of an increased food intake. The normal rat weighing about 250 grams became a giant of 1100 grams. Then they wanted to exclude the pituitary as a cause of this phenomenon, and did hypophysectomy first and then produced hypothalamic damage. These animals ate tremendously following the operation, became obese, but did not increase in length. They looked like butterballs. Food alone doesn't seem to be a sufficient stimulant for linear growth.

I should like to ask a question concerning the assumption by Dr. Randle that the diabetes due to pituitary factors is accompanied always by underutilization of glucose? In a permanent diabetes produced by pituitary factors the same situation will obtain as in pancreatic diabetes, that is underutilization of glucose in the peripheral tissues. Is there really evidence that in the idiohypophyseal diabetes as manifested by hyperglycemia, glycosuria, and possibly ketosis, that there is underutilization, and conversely, is there evidence that hypophysectomy increased the utilization of sugar in the peripheral tissues, apart from the experiments using the diaphragm? I ask this because the hepatectomized hypophysectomized animal with which we worked some years ago (12) (and I think Dr. Houssay has similar experiments on the maintenance of blood sugar), the same amount of glucose as in the normal preparation will maintain the same blood sugar, despite the absence of the hypophysis. There was no evidence of increased utilization in the absence of the hypophysis in the dog.

DR. RANDLE: Young's studies showed that the idiohypophyseal cat and dog show nitrogen retention despite glycosuria (Young, F. G.: *Biochem. J. 39*:515, 1945). Thus there is in this condition a continual loss of glucose in a condition in which gluconeogenesis from protein is reduced, in which protein is being deposited, body weight increased and body fat burned. This would suggest that the peripheral utilization of glucose must be reduced. It is a peculiar sort of metabolic balance and I don't see how it can be explained if peripheral glucose utilization is not markedly reduced. There is

[12] SOSKIN, S., LEVINE, R. AND LEHMAN, W. *Am J Physiol.*, 127:463, 1939.

DR. SAMUELS: I would like to bring up a related point, namely the work that Dr. Roberts did when he was at Minnesota on hypophysectomized and normal rats fed diets of different compositions at constant total caloric and total protein levels He studied the disappearance of glucose in these animals after they had been eviscerated. When the high fat diet had been maintained for a sufficiently long time the subsequent rate of glucose disappearance was greatly decreased. This was not due to circulating acetone bodies or anything of that sort. The actual metabolism in the peripheral tissues, as demonstrated later by Dr. Hanson, had been altered by this difference in diet. We felt that there was some evidence of an adaptive change in metabolic systems. I think that this can enter into the picture in connection with these effects of hormones that we are talking about. If you maintain an animal for some time on an excessive amount of a hormone which increases the utilization of fat, and you test the glucose utilization of that animal later under the influence of insulin you will observe a different picture. This is not necessarily the effect of the growth hormone, but may be just an effect of the adjustment of enzyme systems to the new milieu. I think this enters into many of the problems we think about. We have seen this in regard to the question of ACTH action, where one tests the adrenal at one time with a given level of ACTH, and get a certain response. If the animal is repeatedly treated, however, one gets a much greater response from the same amount of ACTH. I think the change is undoubtedly due to an increase in what we call functional tissue; the enzyme systems that are producing the adrenal hormones. This is probably a compensatory or adaptive enzyme mechanism.

DR. GORDON: There are two other points that I would like to mention. The first is directed to Dr. Best relative to his experiment on glucagon. We discussed last night a little about the possible mechanism. I interpreted this as a modification of the Dohan and Lukens experiment, in which they produce permanent diabetes in cats by the constant infusion of glucose. In this instance, however, the source of the glucose is not from the outside, but from hydrolyzed glycogen in the liver. It is difficult for me to see how the liver can be making glycogen in such huge amounts all of the time and continue discharging it into the blood stream in

blood or in the pancreas. In the child who has had a remission, and requires no insulin, even though he originally may have required 25 to 50 units of insulin a day, have blood or pancreatic insulin determinations been performed?

DR. BEST: Some of you remember experiments done by Boweie, in partially depancreatized dogs, that became diabetic and showed hydropic degeneration of the islets. When these dogs were treated with insulin another biopsy of the pancreas showed complete disappearance of the hydropic degeneration. My memory is not good enough to permit me to tell you whether there was restriction of diet, then a biopsy, and then a full diet plus insulin. As I remember the dog was operated on several times, but certainly insulin alone gave a disappearance of the hydropic degeneration. There was a child among the early patients who was treated vigorously by strict diet and insulin; I believe he died as a result of a motor car accident. At autopsy (no insulin determinations were done in those days), Dr. Robinson, the pathologist, found what he called regeneration of the beta cells. That is the only case of which I know. In Dr. Wrenshall's series, where he has been studying insulin content, there is just the one determination—at autopsy.

DR. STETTEN: In answer to Dr. Randle's question on the quantities of glucose going to fat and glycogen, we can only give a few guesses. In earlier experiment of 12 years ago or more, we concluded that of the total sugar available to a rat on a fat free, carbohydrate diet, some 30% was consumed daily to replenish the fat store. This is while the animal is in approximate nutritional balance. Presumably therefore an equivalent amount of fat would be mobilized and oxidized daily by such animals. As to the utilization of glucose by muscle in the basal rat, rather the sub-basal or anesthetized rat, we were surprised to find in some experiments with Ingle that the carbon dioxide evolved by such animals had a specific activity of maximally about 20% of that in the circulating blood. This would imply that of the carbon dioxide produced, only some 20% was derived immediately from glucose, and the remainder was derived from other sources. We don't know what the other sources are.

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amounts sufficient to produce constant hyperglycemia.

The other point I would like to raise is a biochemical one. This is directed toward Dr. Lardy or Dr. Stetten. First about glycogen being a cul-de-sac. I wonder if it is always a cul-de-sac. There are numerous instances in which glycogen seems to be deposited, not merely because there is nothing else to do with it, but seemingly because it is needed there for some special purpose. One instance that occurs to me immediately is the deposition of glycogen in the endometrium in the lutein phase of the ovarian cycle. In the same connection but in a different direction, I would like to ask a question that might seem bizarre: what are ketone bodies for anyway? They seem to be another form of a cul-de-sac, characteristic of hepatic tissue. It seems that peripheral tissues can utilize two carbon fragments perfectly well. Why, then, does the liver seem obliged to make ketone bodies?

DR. STETTEN: I'm not sure what Dr. Lardy meant by cul-de-sac, but I know what I meant. That is, that the only way out is a reverse of the way in. Thus, it is a cul-de-sac in the same sense that the vermiform appendix is a cul-de-sac. It is hard to see that glycogen, unless it undergoes reactions of which we are completely unaware at this time, can serve any other functions than that of a reservoir. It enters into no other reactions except those that are the reverse of those that formed it. I certainly don't mean to imply that it is functionless.

DR. LARDY: That is essentially my definition of the glycogen cul-de-sac too. In regard to the second part of the question, aceto-acetate seems to be an essential metabolite in certain syntheses. For example there is evidence that cholesterol is synthesized more directly from aceto-acetate than it is from acetic acid. Experiments of Block have shown that the specific activity of cholesterol is higher when labelled aceto-acetate is the precursor. There are other compounds related to aceto-acetate. Animals have varying degrees of ability to synthesize those compounds, and presumably these too would come from aceto-acetate.

DR. STADIE: If we accept the concept, keeping it simple, that the diabetic is essentially unable to utilize carbohydrate in the periph-

ery, there is only one mechanism that I know of that has been established to compensate for that deficiency, and that is for the liver to oxidize fatty acids at a sufficiently increased rate so as to form aceto-acetic and beta hydroxybutyric acid in abundance to supply the periphery with nutrilites for energetic needs. It has been well established that the periphery utilizes these ketones readily. If the liver did not make ketone bodies the diabetic would have to depend entirely upon fatty acids themselves for peripheral metabolic needs.

Dr. Kinsell: It is my impression, Dr. Stadie, that the maximal rate of keto acid combustion which has ever been demonstrated is grossly inadequate to account for the amount of fatty acid oxidation which occurs even under normal conditions. It seems to us most probable that aceto-acetate and beta hydroxy butyrate also are largely "cul-de-sac" compounds; they are formed in excess, in an effort to avoid excess acetate accumulation; and that the blood levels of these compounds represent indirect measuring sticks of the algebraic sum of acetate formation, and acetate utilization, respectively.

Dr. Best: Dr. Gordon referred to the diabetes produced by large doses of glucagon in oil. There is a doubling, at least, of the nitrogen excretion under these conditions. I don't look upon this as just an increase in liver glycogen breakdown, but as increased gluconeogenesis. In further comment upon Dr. Houssay's statement, and perhaps Dr. Samuel's, I think we must use Houssay animals in these studies of growth during constant dietary intake. When one force-feeds in the presence of the pancreas, he never knows how much insulin he is liberating. If you are studying the growth effect of insulin you must have a precise measurement of the amount which is available. Therefore, our further experiments on dogs and rats are all going to be done in animals in which both the pituitary and the pancreas have been removed. By maintaining constant food intake, knowing the insulin intake and the growth hormone intake, and then perhaps varying one at a time, one should get a much better idea of what is happening. There seems little doubt that insulin alone gives nitrogen retention in Houssay cats, dogs and rats.

DR. WILLIAMS: First of all I would like to add to Dr. Best's comments about the effect of glucagon in diabetes by stating that Dr. Kirtley, and also my colleagues and I have shown that some patients given glucagon have an increase in 17-hydroxy-steroids in the urine, although we did find normal levels of 17-hydroxysteroids in the plasma. It has been demonstrated that glucagon may produce eosinopenia. Therefore, I think that Dr. Best's consideration of glucagon causing increased gluconeogenesis is a possibility.

The second point that I wanted to make is in regard to insulin assay. To know how much insulin is produced and how much is circulating would help considerably in explaining many problems in diabetes. I want to point out one or two difficulties involved in the assay of insulin in plasma of diabetic patients, and I would be interested in hearing others comment along this same line.

When one gives labelled insulin intravenously to normal individuals only about 4% of the injected label is in the blood at the end of two hours. However, almost all diabetics who have had insulin treatment for more than a couple of months have an abnormally large retention of label in the blood, sometimes as much as 95%. This binding phenomenon is not specific for diabetics, because schizophrenics who were treated over a period of a couple of months exhibit the same phenomenon; the subject (diabetic or non-diabetic) who has had no previous insulin treatment tends to have a normal curve. This capacity of the plasma to bind insulin excessively interferes with insulin assays. For example, if one places rat diaphragm in a beaker along with insulin-I^{131}, and plasma from normals or diabetics who have had prolonged insulin treatment, much less insulin-I^{131} is transferred to the diaphragm in the beakers with diabetic plasma than in those with normal, showing the greater binding capacity of the former. Most of the assay techniques that have been applied have measured hypoglycemic reactions in various animals, but there is one point that is usually not taken into consideration. When you inject a stock solution of insulin, you get a given depression of blood sugar, but if you will add that same quantity of insulin to one of the above specimens of diabetic plasma you get very little depression of the blood sugar. Therefore you conclude that there is very little insulin present. In our studies we have not yet been able to

split this insulin from the plasma and know whether it is still active or not. There could be a great deal of insulin present. Even with the commonly used assay techniques there have been some patients who have been reported to have either a normal or a hypernormal content of insulin. It seems to me that it is very important for us to improve our insulin assay technique.

DR. ASTWOOD: I would like to go back to one of Dr. Randle's experiments which I found most interesting, showing that a purified corticotrophin preparation would enhance greatly the effect of insulin in causing uptake of glucose by diaphragm. I wonder first of all if this is the same phenomenon that has been noted in mice in which these preparations cause a lowering of the blood sugar? This same preparation of course causes fat mobilization, ketosis, a drop in respiratory quotient, and may be the same thing which Dr. Collip has described as the "Specific Metabolic Principle." All five laboratories who have published the isolation of what appears to be pure corticotropin have obtained material which exhibits these specific metabolic effects. The first question is whether the effect Dr. Randle has studied is the same thing. The second question concerns the remarkable finding that this corticotropin retained its activity after oxidation whereas it has been shown that all corticotrophic action is rapidly lost upon treatment with perioxide or periodate.

DR. RANDLE: It has yet to be established whether the *in vitro* action of corticotropin in enhancing the action of insulin on the glucose uptake of rat diaphragm is related to its hypoglycemic action *in vivo* Our experiments do not establish such a relationship. We have also studied the influence of serum proteins such as serum albumin and they do not share the action of corticotropin on the response of the isolated rat diaphragm to insulin. I was struck last night by the remark of Dr. Williams that corticotropin can inhibit the inactivation of glucagon by what we should perhaps call insulinase. These results of Dr. Williams are similar to those obtained by Dr. Kenny in Baltimore who also found that corticotropin would inhibit the inactivation of glucagon. My present tentative explanation of the action of corticotropin in en-

hancing the action of insulin on the uptake of glucose by the rat diaphragm is that corticotropin inhibits the inactivation of insulin, but I have no proof for that at the present time. I certainly wouldn't suggest that there is necessarily any relation between this *in vitro* action of corticotropin and its hypoglycemic effect *in vivo*.

I should like to ask Dr. Astwood two questions in return. Do you know whether the hypoglycemic activity of corticotropin persists after its ACTH activity is abolished? Do you know also whether corticotropin, like growth hormone, is hypoglycemic in the absence of the pancreas?

DR. ASTWOOD: The hypoglycemic and fat mobilizing properties are abolished, along with the corticotropin action, after peroxide.

V

THE THYROID IN RELATION TO ENERGY METABOLISM

By Dr. J. Gross

Fundamental to any discussion of hormonal metabolic effects are two questions. First, what is the nature of the hormone, and secondly, what is the distribution of the hormone in the body of the animal? From this information it is possible to determine at least the possible target sites for hormone action and then relate this distribution to a metabolic activity. In the case of the thyroid hormone my own experience has been limited to attempts to answer these metabolic questions with little first hand experience on the fundamental mechanism of thyroid hormone action.

I should like to spend a short period reviewing the material that is known about the nature of the thyroid hormone, and especially to indicate what is known about the distribution of the hormone. From the latter it may be possible to speculate as to the major pathways of action of thyroid hormone.

The Thyroid Hormone.

We have to consider first thyroxine, then triiodothyronine and, perhaps, triiodothyroacetic acid and 3-3′, diiodothyronine. Of these four, it is reasonably well accepted that thyroxine is the major product formed by the thyroid which enters into the circulation. However, when distribution studies were carried out with thyroxine *it was disappointing to find that the distribution of the* hormone, or at least concentration of the hormone was limited primarily to the kidney and the liver. In the course of these experiments, however, it could be determined that a metabolic product behaving like thriiodothyronine was formed.[1] Since this substance was later found to be more potent than thyroxine, it was suggested that this was the form of thyroid hormone acting on the tissues.

Formation of triiodothyronine from thyroxine in peripheral

tissues has been questioned, especially by Roche and Michel[2] and they suggest that triiodothyronine is merely a by product of thyroxine synthesis in the thyroid. On the other hand there have been several reports[3,4] that would indicate that such conversion can occur in kidney tissue. In order to validate this mechanism I should like to present some data that we have obtained in the kidney perfused *in vitro* with blood.[5] There is an increasing proportion of iodine appearing in the perfusate with time, and when the kidney itself is analyzed, a spot of radioactivity corresponding to triiodothyronine can be demonstrated. In determining the distribution of the radioactivity on the perfusates at various times, a small proportion of radioactivity could be found in the area occupied by triiodothyronine, even at the zero time interval, so the possibility that all the single dimensional chromatograms previously interpreted as indicating the formation of triiodothyronine might have represented the concentration from the medium of pre-existing triiodothyronine, had to be tested. It was found that this percentage of triiodothyronine or triiodothyronine area remained constant or perhaps increased slightly with time during the course of the perfusion. But when two-dimensional chromatograms were run, the nature of this material became obvious. The original labelled material contains, in addition to thyroxine, a material which tends to streak ahead of the thyroxine and encroach on the triiodothyronine area. In the triiodothyronine area, no radioactivity is demonstrated. However, in the kidney the radioactivity spot corresponded well with the triiodothyronine area. On the basis of the possibility of the formation of triiodothyronine from thyroxine peripherally, and its more marked metabolic effects, it is reasonable to assume that its distribution might be indicative of the site of thyroid hormone action

The Distribution of Triiodothyronine as an Indicator of the Target Sites of Thyroid Hormone Action.

Most of the triiodothyronine distributes itself between the liver and carcass of the animal following its intravenous injection. However, the nature of the curves would indicate that the radioactivity entering the carcass must have first passed through the liver, since the peak activity in the liver curve occurs very much

sooner than that in the base of the carcass. Similar curves were found in most of the other organs and tissues investigated and are exemplified by the curve for the pituitary.

Let us now look at a distribution of radioiodine following the administration of labelled triiodothyronine. Most important concentrations occur in the pituitary and the adrenal with lesser, but still significant concentrations occurring in a number of other active organs, for example, in the submaxillary gland, the ovary and pancreas.

Let us consider first the pituitary and its associated neural structures. In the case of the monkey and the rabbit, the concentration in the posterior pituitary always exceeded that in the anterior portion of the gland. In the rat, concentrations were about equal, while in guinea pigs they were reversed and in favor of the anterior pituitary. On examining the hypothalamic region of the brain it was found that the regions of the infundibulum and the mammillary body showed the highest concentrations. When these areas were isolated this concentration became even more evident in the rabbit, monkey, and the guinea pig.

We now have evidence of the entry into and the concentration of a thyroactive substance in the complex that is associated with pituitary hormone formation and secretion. You will recall that it is lesions in the posterior part of the pituitary (i e., in the region of the mammillary body) which tend to interfere with the secretion of adrenocorticotropin. We have then the possibility of implicating by association thyroid hormone with the formation and production of the pituitary hormones. Similarly, analyses of the adrenal by separation into cortex and the medulla, indicated that for the most part, the concentration was greater in the medulla than in the cortex, but that in both cases it was high. This may indicate involvement of the thyroid hormone in the production of either the cortical and/or medullary hormones of the adrenal. We have not yet been able to analyze the localization of hormone in the pancreas with regard to the islet cells or the exocrine tissue, but since there is a rather marked concentration in the salivary glands, it may be implied that the relative concentration in the pancreatic islets is unlikely to be much different from that in the enzyme secreting tissue.

THE IMPLICATIONS OF THE DISTRIBUTION PATTERN OF THE THYROID HORMONE IN RELATION TO THE POSSIBLE SITES OF ACTION

General Behavior of Thyroid Hormone. When a single dose of thyroid hormone is given to a hypothyroid individual there occurs a prompt rise in the oxygen consumption which reaches its peak in a day or two, and slowly falls (half time eight days). A similar phenomenon occurs in relation to nitrogen balance and creatine excretion. If, however, one attempts to correlate this course of effect with the curve of thyroid hormone itself one finds that in the case of triiodothyronine the active substance very rapidly disappears from the circulation, with a half-time of 2.4 days for total radioactivity. In some of our own experiments (Bell, Friedman, Symchowicz and Gross, unpublished) in which the plasma radioactivity has been fractionated, the fall of triiodothyronine is more rapid. (Only 10% of the injected triiodothyronine remains in the circulation after five hours.) A corollary of this phenomenon is the decay in metabolism following the removal of the thyroid gland or the cessation of thyroid therapy. This would suggest that the metabolic changes demonstrated by thyroid hormone are an indirect rather than a direct effect. Cytochrome oxidase, cytochrome c and succinoxidase[6] which increased under the influence of thyroid hormone treatment, fail to do so in the absence of an adequate amount of adrenal-cortical secretion. This would tend to explain the relative lack of success of the demonstration of thyroid hormone effects when the substances are added to tissue preparations *in vitro*. Nevertheless the fact that the thyroid hormone can be demonstrated to be present even in small concentrations throughout most of the body tissues, does not preclude a direct effect on the enzyme system, although it might exert its action at these sites by the potentiation or inhibition of another active substance.

Indirect Effects.

From the distribution data it would seem reasonable to consider that the indirect effects of thyroid hormone might be mediated through the hypophysis, the adrenal cortex, the adrenal medulla, and possibly the pancreas.

HYPOPHYSIS

Growth: One of the common responses to thyroidectomy, as is well known, is a depression of growth. This, incidentally, is accompanied by a disappearance of the acidophil cells of the pituitary (implicated as the producers of growth hormone). Conversely, treatment with thyroid hormone in restoring growth also restores the acidophils. This would suggest perhaps that thyroid hormone increases the production of growth hormone, and/or that thyroid hormone potentiates the action of growth hormone. The latter effect has in fact been shown to occur, in that the response of thyroidectomized rats to growth hormone is rather small.[7] Similarly, in the hypophysectomized-thyroidectomized animal, the response to growth hormone is very much reduced; and as a corollary as little as 0.25 micrograms of thyroxine almost triples the response to growth hormone in the tibial line test.[8] In addition the overall growth in hypophysectomized-thyroidectomized animals is virtually zero.

In the hypophysectomized animal thyroxine can cause some increase in weight, in food intake, and in the proportion of carcass weight as protein.[9] Under the same conditions insulin also caused a weight gain,[10] with a relatively greater increase in carcass fat. Extending this work, Salter and Melgaard, as quoted by Best,[11] found that the effect of insulin and thyroxine together resulted in a marked increase in growth, which was due to a rapid synthesis of total body protein. These results would indicate a primary effect of thyroid hormone (possibly in conjunction with insulin), on protein synthesis and growth, and that growth hormone potentiates this effect. In addition, there is the possibility that thyroid hormone acting on the hypophysis produces an increased secretion of growth hormone, thus multiplying the growth effect.

Corticotropin and the Adrenal.

In the thyroidectomized animal, the adrenal shows a decrease in the zona fasciculata[12] However, there is no change in ACTH content of the pituitary of the thyroidectomized animal[13] (Halmi and Bogdanove, 1951). Treatment of the thyroidectomized or normal animal with thyroid hormone results in increased size of the adrenal, which is manifested as cortical enlargement. This

increase does not occur on treatment of the hypophysectomized animal with thyroid hormone. This would indicate that the thyroid hormone can stimulate secretion of corticotropin from the pituitary, and that this stimulation is most marked when the thyroid hormone treatment begins to exceed the normal requirement. It has also been shown that in hypothyroidism, the effect of ACTH on the adrenal is reduced, and that thyroid hormone treatment returns this effect to normal,[14] thus suggesting that in addition there is potentiation of the action of corticotropin on the adrenal. Thyroid hormones may also act to potentiate the action of cortical hormones themselves. This is supported by the experiment of Wells,[15] in which in the hypophysectomized animal thyrotropin plus compound E increased the rate of gluconeogenesis over treatment with the cortical hormone alone. If we accept the possibility that one of the actions of thyroid hormone in the hypophysis is to increase the output and effectiveness of cortical hormones, then it is interesting to determine what metabolic effects attributable to thyroid hormone can be effected by the system. It has been shown that the thyroid-induced increase in negative nitrogen balance,[16] succinoxidase, cytochrome oxidase, and cytochrome c,[6] growth in normal mice;[17] and diuresis[18] are prevented or depressed in the adrenalectomized animal. On the other hand in the human there was some increase in thyroid-hormone-induced oxygen consumption caused by treatment with ACTH or cortisone.[14] This now represents an additional system which may be implicated, especially in the toxic manifestations of thyroid hormone.

Gonadotropins.

In going through the confusing and often irreconciliable literature[19] on thyroid effect on the hypophysis-gonad system, there is some indication that thyroid hormone may maintain or stimulate the secretion of gonadotropins. For example in the thyroidectomized male rat the gonad weight is reduced On treatment with thyroid hormone the weight and functional morphology is returned to normal.[20] The sensitivity of mice to estrone is said to be dependent on thyroid hormone level. Chemical evidence of the association of cryptorchidism with congenital thyroid disease, with

some cures with thyroid hormone treatment; gynecomastia in males; and increased breast size and menstrual irregularities in women with hyperthyroidism, would indicate an involvement, but the locus of action of thyroid hormone is obscure.

Extrahypophyseal Sites of Action.

Treatment of the hypophysectomized animal with thyroid hormone results in a rise of oxygen consumption which is somewhat less than that obtained in the thyroidectomized animal.[25] This would indicate that most of the calorigenic action of thyroid hormone does not require the presence of the hypophysis. Epinephrine might contribute in view of the potentiation of the epinephrine calorigenic action by thyroxine.[21] This is supported by the finding that an adrenergic-blocking agent (Dibenzyline) prevents the calorigenic effect of physiological amount of thyroid hormone (10 micrograms T X/100g) in normal rats.[22] This reaction may be only one component of the calorigenic action, since with large doses (i.e. 50 microgram/100g) the effect of the blocking agent is much less significant. This finding, together with the large body of literature showing a potentiation of epinephrine actions by the thyroid gland on muscle,[cf 21] blood sugar,[23] blood lactate,[24] and creatinuria,[25] would indicate an important role of this substance in mediating some of the metabolic effects attributable to thyroid hormone, as for example the decreased liver, muscle, and cardiac glycogen, elevation in blood and cardiac lactate,[26] and in blood pyruvate[27] found in hyperthyroidism. The potentiation of epinephrine actions may be attributed to the thyroxine induced depression of the level of (a) liver amino-oxidase[28] and (b) adrenolytic enzyme present in blood.[29] There may also be an effect on the production of epinephrine since thyroid hormone has been shown to increase the amount of epinephrine in the glands.[30]

SUMMARY

1. Evidence is presented that of the thyroactive substances demonstrable in the body, triiodothyronine concentrates readily in various organs and tissue. This distribution is taken to indicate the possible sites of action of thyroid hormone.

2. Because of the much longer decay rate for the metabolic

effects, as compared to the decay rate of thyroid hormone itself, it is suggested that these effects are an indirect, rather than direct, effect of the hormone.

3. From the distribution data of triiodothyronine in tissue and from some supporting metabolic evidence it is suggested that many of the metabolic effects of thyroid hormone are mediated through its effect on the activities and secretions of the hypothalamus, the hypophysis, the adrenal cortex, and the adrenal medulla.

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DISCUSSION

DR. STETTEN: May I ask a question in regard to the two dimensional chromatograms of thyroxin, triiodothyronine, and iodide from kidney after perfusion. Do the densities in that radioautograph have any meaning? It seemed rather surprising to me that the iodide spot was considerably more dense than that of the triiodothyronine.

DR. GROSS: The densities of the spots only have meaning relative to the other spots on the same chromatogram. The iodide does escape as shown by the increasing iodide concentration in the perfusate with time. There is some evidence that the spot labeled iodide may contain other substances which follow iodide closely in these solvents.

DR. SZEGO: With all due respect Dr. Gross, I wonder if it isn't a little dangerous to place too much functional significance on accumulation of the isotopic label in various tissues, quantitatively at least. By analogy, other isotopically labelled hormones are very

effects, as compared to the decay rate of thyroid hormone itself, it is suggested that these effects are an indirect, rather than direct, effect of the hormone.

3. From the distribution data of triiodothyronine in tissue and from some supporting metabolic evidence it is suggested that many of the metabolic effects of thyroid hormone are mediated through its effect on the activities and secretions of the hypothalamus, the hypophysis, the adrenal cortex, and the adrenal medulla.

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DR. GROSS: *I wouldn't argue that point, that a site* of localization may represent a site of metabolism.

DR. LEVINE: With the new thyroid materials we seem to have much less of a lag. It is possible to give triiodothyronine or the acetic acid derivative in the hepatectomized animal? Will they have a calorigenic action?

DR. GROSS: I'm sure that it is possible, but I don't know if the experiment has been done.

DR. LARDY: You made a point that probably triiodothyronine exerted an effect which lasted longer than the hormone. What is the half life of the radioactive iodine in the tissue? I am not talking about radioactive decay of the iodine, but about how long some substance other than triiodothyronine may stay in the tissues. I am thinking of further decomposition products or further transformation products of T-3.

DR. GROSS: I can only guess from the data that we have in the rabbit. In the pituitary the concentration reached its peak in two hours, and in 24 hours it was well down below its half value; I would guess that its turnover time is of the order of hours, at least in the rabbit I don't know how it would work in the human.

DR. LARDY: *What do you find with regard to the iodine if you* carry this out for several days? Does it come down to a uniform concentration and then stay there for some extended period?

DR. GROSS: I don't know because we haven't done it. In fractionating the radioactivity at various times in the rabbit, the proportion of triiodothyronine present has remained fairly constant despite the total fall I would expect that had we been forming a secondary metabolite that stayed in the tissues this triiodothyronine proportion would decrease, and the secondary metabolite proportion would increase. With the data that we have, this does not appear to happen.

conspicuous by their failure to concentrate in the typical target organs. This perhaps might emphasize that qualitative rather than quantitative association with target tissues should be sought.

DR. GROSS: I think that everything I have said in relation to concentration is dangerous. I present this merely as a stimulus to discussion and an indication as to where work might be done. It's logical in my mind to look for an action of a hormone in the place where it concentrates. Now as to the data on other labelled hormones, we are in a rather unique position with regard to the thyroid, as we have them in almost "tracer amounts," and we can get an insight into tracer distribution, which I presume is difficult to get with the carbon labelled hormones that you are speaking of.

DR. SZEGO: My objection is based upon what we know about the distribution of iodine-labelled pituitary tropic hormones as well.

DR. GRIFFITH: Fawcett and Kirkwood (Fawcett, D. M. and Kirkwood, S.: Role of the salivary glands in extra-thyroid iodine metabolism *Science, 120*:547, 1954) have suggested that it is a function of the salivary glands to remove excess thyroid hormone from the blood. This conclusion was based on the iodide in the saliva. They believe that a cycle exists as far as iodine was concerned. If this is true, it is one instance perhaps in which the accumulation of iodide in a tissue might be a function of its degradation rather than of its normal function.

DR. ROBERTS: I think that there is merit in the suggestions of both Dr. Szego and Dr. Griffith, that the concentration of a substance in various tissues may better reflect the metabolism of this substance (and usually the degradative metabolism, rather than its physiological action. In work that Dr. Levey and Dr Solomon have been carrying out at UCLA on the distribution of injected TSH in the rat, the greatest amount of the hormone appears in such organs as the liver and in the carcass, some comes out in the urine, and the amount found in the thyroid gland is insignificant. As I recall, the work of Sonenberg with other pituitary hormones points in the same direction.

DR. RANDLE: With respect to the role of the thyroid hormone in the growth promoting action of growth hormone. An action of growth hormone can be demonstrated in the tibia test in the absence of the thyroid (Li, C. H.: *Ciba Foundation Colloquia on Endocrinology*. London, Churchill, *5*:115, 1953).

DR. GROSS: If I can quote Geschwind correctly, in the thyrodectomized-hypophysectomized animals his assay sensitivity dropped to 1/20th of that in the hypophysectomized animal alone.

DR. GROSS: That is true, but equally in the completely thyroidectomized animal, growth hormone produces a very small amount of growth in the whole animal (Scow *et al*, 1949*).

DR. KINSELL: In regard to the sensitivity of the tibial line test, in a seminar conducted by Dr. Asling a while ago, he emphasized that the sensitivity of the test drops most appreciably in the absence of the thyroid. If I may also rise to the bait in this matter of growth as a whole in relation to the thyroid.—One of the things that stimulated this conference was the Growth Hormone Conference held in Detroit about two years ago, at which it became apparent, if it wasn't already so, that nobody really knew what growth was. There were a lot of very interesting questions raised about growth and the hormonal regulation of growth, but very few facts were supplied. One thing that emerged was renewed interest in some of the older concepts to the effect that growth hormone must be assayed in association with other hormonal substances. In the human acromegalic (a subject from whom we can learn much about growth) there is not only evidence of growth, with particular reference to connective tissue, but there are impressive changes in the status of the thyroid, the adrenals, and the islet cells. The previous discussion emphasizes the importance of such hormonal interrelationships.

DR. ASTWOOD: I would like to make a comment about growth hormone and growth. As Dr. Houssay pointed out this morning,

* Scow, R. O., Simpson, M. W., Asling, C. W., Li, C. H. and Evans, H. M.: *Anat. Rec.*, *104* 445, 1949.

DR. LARDY: Triiodothyroacetic acid is a likely transformation product. We have had only small amounts of it to do experiments with, but the propionic acid analogue has been synthesized, and we have found it to be very active in the rat, both in goiter prevention and in stimulating basal metabolic rate. With kidney or liver mitochondria it gives a nice stimulation of respiration in concentrations of about 10^{-6} molar. This is a system somewhat analogous to that which Dr. Thibault and Dr. Pitt-Rivers use, except that they used kidney slices, and measure enhanced respiration.

DR. GORDON: You indicated that there was potentiation of growth in a hypophysectomized animal treated with growth hormone, by as little as a microgram or two of triiodothyronine. I was wondering if that was what one might regard as a permissive effect of thyroid hormone on growth, or is it the other way around?

DR. GROSS: This refers to data of Geschwind in relation to the tibial line test for growth hormone. In this case thyroid hormone would appear to exert a permissive effect. However, since some growth in the hypophysectomized animal can be produced by treatment with thyroid or thyroid and insulin, it may be that the relationship is the other way around. The primary effector may be thyroid.

DR. ROBERTS: I am very much interested in the suggestion that thyroxine may be essential for maintaining the growth hormone secreting activity of the pituitary. It is true that after thyroidectomy there is a degranulation of the eosinophiles, and this can be produced very rapidly by the injection of goitrogens as well. Very quickly after the administration of thyroxine the cells begin to return to normal. In our laboratory it has been observed that thyroidectomy results in a great decrease in the amount of the major protein present in adenohypophyseal extracts, subjected to paper electrophoretic separation. This protein can be restored in a very short time by the intravenous administration of thyroxine. It may be derived from the eosinophils. The protein can also be depleted by the administration of goitrogens. We don't think that this is all growth hormone of course, but we are interested in trying to assay this area on the paper strip for this hormone.

DR. STETTEN: Has anyone ever assayed these insulins in the animals from which they were derived? I suppose that this has been done, but I do not recall having seen it.

DR. BEST: Human insulin has been given to the human, but I don't think it has ever been done as an assay, i.e., compared carefully with insulin from other species.

DR. ASTWOOD: I would like to ask Dr. Gross whether in his investigations of the literature he has encountered the matter of epinephrine affecting the thyroid itself. There have been reports in the clinical literature of sudden thyroid enlargement during intravenous infusion of epinephrine. Others have published data suggesting, on the basis of thyroid weight and iodine uptake, that there is stimulation of the thyroid gland by epinephrine.

DR. GROSS: I believe there is a paper by Jensen (1) Botkin, A. L. and Jensen, H.: *Endocrinol., 50*:68, 1952, which indicates that epinephrine causes an increased peripheral utilization of thyroid hormone in the rat. What is the mechanism of this epinephrine action on thyroid?

DR. LARDY: You indicated that the adrenal was largely responsible for stimulating metabolic rate, and that the thyroid was acting indirectly through the adrenal. What's wrong with the other way around? Have you any evidence that the adrenal hormone is the more directly effective agent than the thyroid hormone? This is referring to cortical hormones, not medullary hormones.

DR. GROSS: I think that Dr. Astwood has some information on the calorigenic effect of the cortical hormones and corticotropin-like substances. He might deal with the question. I can only recall the effect of ACTH or cortical hormone on basal metabolism in a hypothyroid patient while on a constant thyroid intake.

DR. ASTWOOD: I think that what Dr. Lardy meant was that in your diagram perhaps you have indicated that thyroid affected the adrenal cortex which in turn affected the metabolic rate, whereas

in man and in the Rhesus monkey, growth hormone preparations have been remarkably ineffectual. Drs. Greep, Knobil, and Wolfe are testing monkey pituitary growth hormone in monkeys. I don't have permission to quote their preliminary results, but they are very striking. They obtained some 1500 intact pituitaries and permitted Dr. Raben and me to fractionate them, and then they tested the growth fraction in monkeys on carbohydrate and on protein metabolism. They found it to be fully effective. Formerly they had tried growth hormone preparations from pig and beef, and found them not to be effective. The monkey preparation restored the insulin and glucose tolerances of the hypophysectomized monkey to normal, and produced changes in the cartilages of the ribs which Dr. Greep tells me are unmistakably pronounced. We hope of course to try this in man if you can suggest where we can get enough human pituitary to do so. If this proves out, this will remove one source of confusion about growth hormone, i.e. why it seems to be so effective in some animals and ineffectual in others. At the same time, however, it is a most distressing thought if one considers the difficulties that this is now going to get us into, not being able to use pituitary material from one species into another.

DR. STETTEN: May I inquire Dr. Astwood, is there any similar information of insulin species specificity? That is, is beef insulin more effective in the cow and pig insulin more effective in the pig than in other species?

DR. BEST: I don't think so. I think the lack of species specificity is a unique characteristic of insulin.

DR. STETTEN: But there are structural differences. This might be reflected in the biologic stability of the insulin in these various species.

DR. BEST: That is so, but the crystaline material from various sources, about 25 or 30 sources, is of exactly the same potency, but of course, this does not answer the question of species specificity.

with myxedema. Many others have noticed the decreased output of 17-hydroxysteroids and 17-ketosteroids in the hypothyroid patient. When these patients with myxedema were treated with the thyroid hormone they showed a brisk increase in basal metabolism, but there was a very slow return of adrenal cortical function as measured by steroid excretion. These observations suggest that thyroid hormone has direct actions which are not dependent on an entirely normally functioning adrenal cortex.

DR. SAMUELS: I would like to make one additional comment in this regard. In hyperthyroid individuals the "apparent distribution volume," the volume in which the injected cortisone would be distributed at plasma levels, is significantly reduced at the same time that the rate of removal of the cortisone is considerably increased. The actual production necessary to maintain certain levels therefore, is not markedly increased. Apparently, there is a reduction in uptake of cortisol by certain tissues. This does not seem consistent with the general effect of thyroid hormone. This apparent change in distribution volume is a consistent finding.

DR. LARDY. Was it of a sufficient order of magnitude to account for the enhanced effectiveness of the adrenal cortical hormones? That is, might it be keeping it out of one place and making it more effective in other places?

DR. SAMUELS: I think so.

DR. LEVINE: Has epinephrine ever been shown to affect tissues *in vitro* from the calorigenic standpoint, as well as diminish the P:O ratio?

DR. LARDY. I don't think that it has been shown to decrease the P:O ratio by uncoupling. It may function as a shunt in hydogen transport between the DPN or flavoprotein and cytochrome C. There are many studies which have been done on epinephrine *in vitro*, but I don't recall the details any longer. I do know that diaphragm slices do not respond by increased respiration even though glycogen is broken down and hexosmonophosphate accumulates.

wouldn't it be just as reasonable to suppose that adrenal cortical hormones are essential, or permissive in Ingle's sense, for thyroxine to act on tissue?

DR. GROSS: The diagram was meant to indicate that part of the calorigenic response to thyroid hormone is mediated by its effect on adrenal-cortical secretion.

DR. SAMUELS: In regard to that matter we have carried out an experiment which we have never repeated and therefore have not reported, though the results at the time were quite definite. We hypophysectomized animals, and gave one group thyroxine while the other received none. Then we gave a constant daily dose of ACTH to both groups and tested the effect on the size of the adrenal. In the thyroxine-treated animals the increase in size of the adrenal for a given ACTH stimulus was considerably larger, so the increased metabolic rate was also helpful in this local growth response. There is also another factor in the relationship between the thyroid and the adrenal, I think. When we studied the levels of adrenal steroids in thyroidectomized animals we found them low, and also when we tested the rate of destruction, both in humans and in thyroidectomized dogs, the rate of removal of adrenal steroids was considerably reduced. When the level of thyroid function was increased above normal there was a more marked increase in the rate of destruction than in the rate of production of the adrenal hormones. It seems then, that this somewhat differential influence may affect the level of adernal steroids acting on tissues. Thus, there can be an indirect effect of the thyroid. Dr. Gross has been very cautious in his statements about triiodothyroacetic acid, and I gather that he has not had the same experience as Mrs. Pitt-Rivers has had. I would be interested in his comments.

DR. GROSS: I have had no personal experience with the metabolic effects of triiodothyroacetic acid.

DR. LUETSCHER: I was interested in the observations of Dr. Samuels on his animals. We have made similar observations in patients

DR. GORDON: Is this the same compound that you and Dr. Larson and Dr. Albright are working on?

DR. LARDY: No, it behaves entirely differently.

DR. ASTWOOD: I wonder if Dr. Gross would care to comment about the recent finding in Chaikoff's laboratory of triiodothyronine in thyroid vein blood.

DR. GROSS: I think that this is an expected phenomenon, since triiodothyronine has been demonstrated to occur in the thyroid.

DR. ASTWOOD: Do you feel that the thyroid may synthesize triiodothyronine and excrete it as a part of its normal secretory process?

DR. GROSS: In the normal secretory process probably relatively little triiodothyronine is synthesized and secreted as compared to thyroxine. Under conditions in which there is iodine deficiency the relative proportion of iodine going into triiodothyronine as compared to thyroxine is much increased. It may be that the animal under conditions of iodine deficiency may tend to form more triiodothyronine than thyroxine, which would be a more efficient system.

DR. LARDY: There is a recent paper in the French literature that describes just that. Under conditions of iodine deficiency the product from the thyroid was proportionally greater in T-3 than in T-4, and the contrary was the case when iodine was supplied abundantly. I don't remember the name of the investigator.

DR. HOUSSAY: Dr. Gross, in a recent paper by Roche and Michel, they say that there are three hormones secreted from the thyroid, thyroxine, diiodo, and triiodothyronine that are found in blood. Until now then we know that there are three hormones produced by the gland, and are found in the blood.

DR. GROSS: The third compound was the one that I mentioned at the beginning, 3:3-diiodothyronine. There has been little further

Dr. Roberts: As far as I know, the only tissue whose respiratory metabolism can be stimulated by epinephrine *in vitro* appears to be the anterior pituitary gland. On the other hand, the *in vivo* administration of epinephrine has been reported to result in a stimulation of respiration in various tissues, including brain tissue (*cf.* Roberts, S. and Keller, M. R.: *Endocrinol.* 57.64, 1955).

Dr. Levine: Has adrenochrome been tested in various tissues for its effect?

Dr. Gross: I think that Tebow tried adrenochrome directly and didn't get any effect.

Dr. Roberts: I would suspect that there would probably be no effect, because in these studies on the *in vitro* effects of epinephrine on anterior pituitary tissue, when the typical color of adrenochrome appeared the stimulatory effect was lost.

Dr. Lardy: Dr. Gross, would you tell us about the transport compounds?

Dr. Gross: We have some evidence for the formation of a transport form of triiodothyronine by the liver.

Dr. Lardy: I think it would be very interesting if a transport form of triiodothyronine were to exist. It has been pointed out that there are extremely low concentrations of free triiodothyronine in blood plasma and they don't seem to be closely related to thyroid function. Perhaps there is an obscure form that we haven't been looking for. It may be that this will give added strength to the position of those who contend that triiodothyronine is the cellularly active form of the hormone.

Dr. Gross: *If it is true I think it will explain,* as you point out, the difficulty in consistently demonstrating T-3 in the plasma and would also support Roche's contention that what we do see in the plasma is T-3 derived from thyroid. I think that the T-3 derived from thyroxine may exist in the circulation in this transport form. This is still to be demonstrated.

ring effect the dissociation of the phenolic group. Another point I would like to raise is concerning the relative effect of several of these compounds. This is seen especially in the tadpole assay, where larger numbers of animals can be used and more accurate results obtained. We used the assay of Bruice, Winzler and Kharasch, in which the log of the dose of thyroxine or any other agent is plotted against the decrease in tail width. If we plot the data for thyroxine, they form a straight line, and all the other analogues give lines that are nicely parallel with that of thyroxine and falling either to left or right, depending on whether they are more active or less active than thyroxine. However, there are two exceptions. Triiodothyronine and triiodothyropropionic acid give an entirely different slope. They are much more effective at low concentrations than thyroxine is, and become relatively less effective as the dose is increased. Therefore, one cannot say that triiodothyronine is five or ten times more active in the tadpole because there is no definite ratio. We have studied the tetraiodoproprionic acid compound which Bruice, Winzler and Kharasch reported to be 130 times as active as thyroxine in the tadpole. We have used exactly the same assay but a different species of tadpole, Rana Clamitans (they used Rana Catesbiana). Our data indicate that the tetraiodothyroproprionic acid compound is 20 times as active as thyroxine. This is notably less than Bruice and Coworkers found for their species. The most active compound that we have assayed is the triiodothyronamine which is about 35 times as effective as thyroxine. It would be very interesting to see what that compound does in Catesbiana. One cannot predict the relative values of these compounds in different amphibian species. We have some compounds that responded in our assay, which were ineffective for Winzler. Conversely they had some compounds of low activity that gave better ratios of activity in our assay with R. Clamitans.

DR. WILLIAMS: I would like to ask Dr. Lardy which of these compounds is effective in causing an immediate increase in respiratory quotient. That is, in tissue slices.

DR. LARDY: The propionic acid compound will give an immediate response in mitochondria. We take that to mean that it gets into the slice or the mitochondria very rapidly, because of not having

work on this compound and at the moment I wouldn't like to speculate as to the sequence involved and as to its importance in the system. On a functional basis this compound when given to the animal is about as effective as thyroxine but not as effective as triiodothyronine. So far as I know, the most effective substance in replacing thyroid hormone is triiodothyronine. I am not sure but what Dr. Lardy has some compounds that are equally or more effective.

DR. LARDY: I want to use the blackboard for a moment. The assays that have been done on the mammal indicate that if thyroxine is assigned a relative activity of one, triiodothyronine has a relative activity of five to ten, and with Roche's new hormone, which contains only one iodine atom in each ring there is also an index of activity of nearly one. Roche and Michel made the other possible triiodo compound, that is with one iodine atom in the first ring and two iodine atoms in the second ring, and this had an activity of five percent of that of thyroxine, 0.05 on this relative scale. Many years ago Harington and more recently Nieman, had synthesized the diiodo compound with no halogens in the first ring and two halogens in the second, and had shown that it was inactive. When Roche published that the 3,3'5' triiodo compound had such low activity, and that the 3,3' one had fairly good activity, it occurred to us that perhaps the deiodinating enzyme had the same specificity as the plasma component that binds thyroxine. That is, it needs four iodine atoms to bind. Possibly, the reason the 3,3',5' compound was so ineffective was that it lacked the additional halogen atom in the first ring, which might be required, to combine with the deiodinating enzyme. Then the question arose is there any metabolic function for the halogen atom in the first ring at all? Perhaps only one halogen atom in the second ring is sufficient for hormone activity. Dr. Tomita in our laboratory has synthesized this 3' monoiodo compound. Unfortunately it was found to be completely inactive in the tadpole, in the goiter prevention, and basal metabolic rate tests. Therefore, there is a function for the halogen atom in the first ring. I think that is important, because the halogens in the first ring do not influence the activity of the phenolic ring. Their effect is not transmitted through the ether linkage. Only the halogen atoms in the second

pared with that of the guinea pig, may not be that at all. The pituitary of the rat weighs approximately 10 milligrams, and it is hard to imagine it secreting in 24 hours 10 milligrams of the tropic hormone. This may be an analogy to the situation with growth hormone. Dr. Gross, is there any species specificity in thyroid hormone? Many workers have had trouble in finding a reason why fish have a thyroid gland. The fish thyroid responds by goiter formation under certain circumstances, but thyroid hormone, as we know it, when given to a fish, doesn't do anything.

DR. GROSS: Gorbman, as well as Leloup and Fontaine in Paris, has been able to demonstrate thyroxine in the blood of fish. Unless the specificity resides in the binding protein of the plasma it is hard for me to see how the activated iodinated group can possibly be species specific. Why it doesn't work in fish I do not know.

DR. HOUSSAY: In the paper of Fontaine he mentioned the role of the thyroid in the maturation of some fishes, such as the salmon. Regarding the question of the specificity of some hormones, usually there is very little specificity in most species, but one notable exception is the case of gonadotropins. Growth hormone of fishes is very inactive in mammals, and has very little diabetogenic action also, but in the fishes' does have the action that you mentioned. I studied the diabetogenic action many years ago, and now Wilhelmi has studied that in relation to growth (1940-1941). The gonadotropin of batrachians is extremely inactive in mammals. In mice, injected with 100 mg. of toad pituitary, there is no sexual stimulation. One milligram of toad pituitary injected in the toad gave a definite action. The reverse is true as well. When one injects into a frog or toad the pituitary of a toad there is ovulation in response to very small quantities. Thirty or 50 units of chorionic gonadotropin are needed in the toad, but only one, or two or three units in the mammals. Thus, in the case of gonadotropin there are many instances of some specificity though not complete, and in growth hormone there is species specificity in monkeys, in fishes, and in man. There is no question of that.

I have one further comment about the action of thyroid and pituitary on growth. In the animal deprived of both glands,

the amino group. We have uniformly found that compounds without the amino acid side chain give immediate responses in mitochondria preparations, whereas those that do have amino acid side chains require a preincubation period or higher temperature before their effects are apparent.

DR. GRIFFITH: Dr. Lardy, what is the simplest system from the standpoint of tissue or cellular architecture in which you can find an increased oxygen consumption in testing any of these compounds?

DR. LARDY: Mitochondria with an oxidizable substrate and without a phosphate acceptor is the system which has the fewest components in it. The kidney slice may transform triiodothyronine to a physiologically more active compound. If so, it might be a better test system than the mitochondrial preparation.

DR. KINSELL: Going back to Dr. Astwood's comment about species specificity. In humans, the same "growth hormone" that doesn't produce growth, almost without exception contains some thyrotropin, very small amounts to be sure, but amounts enough to produce significant and predictable results. This might suggest that the lack of growth effect is probably not on an immunologic basis.

DR. ASTWOOD. The human thyroid appears to be extraordinarily sensitive to thyrotropin, and growth hormone preparations made by neutral or alkaline extraction do seem to have thyrotropin contaminating them. This has been shown by animal assays as well. I am trying to think of some analogy to the relative ineffectiveness of beef or pig growth hormone in primates. The rat is highly resistent to the thyrotropin derived from slaughter house animal pituitaries. On an individual basis I would guess that man is more sensitive to thyrotropin than a rat. It may take 10 milligrams daily of a preparation of thyrotropin in a rat to double the size of the thyroid in several days, but the same dosage in man may have quite striking effects. It may be that what we formerly thought of as unresponsiveness of the thyroid of the rat, as com-

fact that one can, by depressing thyroid function in the rat, usually with propylthiouracil or with propylthiouracil plus thyroxin, render the rat much more sensitive to the effects of TSH.

DR. LARDY: In our laboratory Dr. Richard Doisy has confirmed the results of Hoffmann. He too finds that if the adrenals and the thyroid are removed from an animal, injected thyroxin gives an increase in basal metabolic rate only if the animals are receiving maintenance doses of cortison. I'd like to mention also that Brewster has shown that thyroxin does not give an increase in metabolic rate if the animal is under a complete sympathetic block. I don't remember the agent that was used but it was described in the Federation Proceedings of April 1954.

Dr. Astwood, would you be able to explain the extreme resistance of the rat to parathyroid hormone on the basis of species specificity? Has rat parathyroid hormone ever been administered to a rat?

DR. ASTWOOD: I am fairly sure that rat parathyroid hormone has not been prepared Dr. Munson and co-workers have shown that if a rat is given a low calcium diet for four days before the parathyroids are removed, such an animal is very sensitive to parathyroid hormone, requiring only two to 10 units to cause an increase in blood calcium in six hours. Similarly Talmidge and his co-workers have shown that immediately after parathyroidectomy the phosphate excretion in the urine is about one-fiftieth of normal, and only about one unit of parathyroid hormone is needed during that period to cause an increase in phosphate excretion. We too have found that the quantity of calcium in the diet affects the sensitivity to parathyroid hormone The tendency to regard the rat as very insensitive to parathyroid hormone depends upon the conditions of the experiment.

DR. GORDON: There has been a lot of discussion about the species concentration of growth hormone. If one plots the concentration of growth hormone in the pituitary gland against age, what sort of a curve does he get? It seems logical that one would expect a higher concentration, or at least a higher discharge of this hor-

growth hormone apparently has more action alone than does thyroid hormone. An additional important fact is that the tissues which develop in response to each of the hormones are different. I believe that it has been shown that growth hormone induces better stimulation of the collagenous tissues.

DR. GROSS: The skin and hair grow particularly well in the thyroxine treated hypophysectomized animal.

DR. HOUSSAY: There is a synergy between somatotropin and thyroid hormone on body growth and this has been described many times in the literature. Furthermore the stimulating effect of thyroid hormone on protein catabolism and on calorigenesis is reduced or absent in the animal without adrenal glands. The experiments to which I am referring were done in adrenalectomized animals which, when maintained with a very small amount of cortisone, got a markedly increased calorigenic effect. This very small amount of cortical hormone, inactive alone, allowed the thyroid response. This is a very striking case of what one would call conditioning or permissive action. There is always the possibility that this calorigenic response is mediated through epinephrine, which should be eliminated by adrenalectomy, but even with the adrenal medulla removed there is a large amount of epinephrine in the body from other sources.

DR. GROSS. There is a possibility also that you might have epinephrine available from sources other than the medulla, and that's why it's such a difficult question to test experimentally.

DR. ROBERTS: I am very much intrigued with the problem of specificity of the protein pituitary hormones and their different quantitative effects in different species. It is very difficult to dissociate this aspect of the problem from the variable sensitivities of different types of animals. For example, one of the points that you mentioned, was the fact that the rat is very insensitive to TSH. It is thought by some that the rat pituitary produces relatively enormous quantities of TSH, and therefore the thyroid gland is relatively insensitive to TSH. This is perhaps borne out by the

when the fetal pituitary is not necessary for development. Some weeks after birth, pituitary hormone becomes necessary for normal growth.

DR. KINSELL: Humans who have had their pituitaries removed, and who are maintained on target organ replacement therapy, are kept in an excellent state of health, in terms of all usual criteria. That is not a complete answer, but is perhaps part of an answer to this question. In any event it is not absolutely essential to reasonable health.

DR. WICK: I have a question for Dr. Lardy. In your assay method *you said you had a problem of cell permeability. If that is so, how* do you know that your assay method is correct?

DR. LARDY: We don't. In the tadpole assay, you put the substance to be assayed in the water and depend upon the tadpole's skin, and chiefly the gills, to absorb the compound. The difference in activity between compounds certainly reflects to some extent the differences in absorption. You can inject the compounds into the tadpole but we have never been able to do that quantitatively. As soon as you pull the needle out the tadpole squirts out whatever you have injected in. As Dr. Gross pointed out last night you can get very specific actions by implanting thyroid hormone at certain sites. I don't think that injection would be better than the technique that we use.

mone during the growing period than during adult life. I would also like to ask the correlated question, what evidence is there, either experimental or clinical that there is a function for growth hormone in adult animals?

DR. HOUSSAY: The name "growth hormone" is interesting historically, but is not the best name. Growth hormone has many other metabolic actions, in addition to growth. The growth phenomenon was demonstrated in the first experiments of Long and Evans in rats and that is why it is called growth hormone. We know that this hormone has actions on fat, protein and carbohydrate metabolism, and so really the name should be something other than growth hormone. This is also the case with many other hormones which have names that produce confusion in our minds. Growth hormone is one with many metabolic aspects of which growth is just one.

DR. GORDON: I realize that we are involved in a problem of terminology but there is a certain common understanding as to what we call "growth hormone"; we might label it "somatotropin" if you prefer. Is the substance prepared by extraction, higher in concentration in the pituitary glands of young animals than it is in adult animals?

DR. HOUSSAY: Concerning the action on growth and pituitary function in the fetus, I don't remember whether or not there has been a determination of growth hormone in the pituitary gland during fetal life but determinations of diabetogenic action have been done. It begins very early. If both actions are the same then we can assume growth hormone to be present very early also. But one may destroy the pituitary, as done recently in the experiments of Jost by decapitation of rabbits in utero, and fetal growth can proceed normally without the pituitary. If hypophysectomy is performed the first day after birth, during the next two to three weeks growth is perfectly normal. Thus, we have another demonstration that the hormones are regulatory agents of a function existing before the hormones. Growth is present before the pituitary is developed in the fetus. Growth is also present apparently

which turned out not to be of any particular help, but the working hypothesis involving degeneration was essential from many points of view. Without this motivation there would have been no start in Toronto. Simple procedures, such as immediate freezing of the pancreas, and mincing it while frozen were helpful. They were used because we felt that we must try to avoid the action of proteolytic enzymes. Also the concentration of alcohol was determined on that basis, the same concentration that is used today for the extraction of insulin. My friend and classmate, Henry Borsook helped me run the scale of alcohol to determine when all proteolytic enzymes were precipitated. Sixty-six percent of alcohol, was the concentration which I adopted. We also used acid, which gave a pH of 1.5 in the mixture. We have repeated this experiment recently and have found that this is an excellent pH for the extraction.

In the autumn of 1921, we were on the horns of a dilemma, whether to pile up more evidence for the presence of insulin, or to block out new fields for ourselves. During the summer of 1921 *in 10 diabetic dogs, and on 75 separate occasions, without any* exception, we had recorded a definite fall in blood sugar. We felt that we had to do this, because Professor McLeod had been very skeptical. If you read the original edition of his book, you will see that he piled up the evidence on both sides, and rather leaned toward the conclusion that there was not an internal secretion of the pancreas.

We first produced hypoglycemia with insulin, on August 14, 1921, using 30 cc's of extract, intravenously. We recorded in our notes that a deliberate attempt was made to lower blood sugar below the normal level, and it did go below the normal level. The dog showed symptoms of hypoglycemia, from which he recovered spontaneously. On November 8, 1921, we recorded in our notebook that a dog felt much better after the administration of sugar. This dog had received saline extract of degenerated pancreas. We found that fetal calf pancreas has an insulin content 17 times that of normal beef pancreas. The insulin content is very high during intrauterine life, falls somewhat, but is still high at birth, and then gradually falls off during life. Simple extraction, with acid alcohol, gave us many times as much insulin

VI

INSULIN—REMINISCENCES

By Charles Best

I have been asked by a number of the members of the sympo sium to say a little about the early work on insulin. I have always been fascinated to hear Dr. Houssay reminisce about the earlier work which he did in relation to the anterior pituitary. Dr. Joslin, of course, didn't reminisce. He looked ahead.

I have had occasion recently to go over all the papers in which attempts were made to prepare antidiabetic hormone from the pancreas since the original publication of Von Mering and Minkowski. Some of the investigators had small amounts of insulin in their extracts, and others narrowly missed demonstrating a potent antidiabetic substance. The paper of E. L. Scott has always fascinated me because he used alcohol in a concentration in which insulin is insoluble. He did get some active material in his watery extract. Dr. Zuelzer's experiments are of tremendous interest. He sometimes ground the pancreas with alkali, and I think could have had little insulin, but in other experiments he probably did have appreciable amounts. He used two depancreatized dogs only, and observations on them were separated by an interval of two years. He probably got in trouble with the testing of his substances against the rise of blood sugar caused by epinephrine, and of course he did not have available an accurate micro method for blood sugar determinations. Dr. Kleiner certainly had some active material, as did Dr. Paulesco. Dr. Paulesco was plagued with a bad method for blood sugar determinations and his figures were all over the lot. This may have delayed his work which really was contemporary with that which Banting and I did.

You all know that Fred Banting independently formulated an hypothesis about ligating the pancreatic ducts. This, we soon learned, was not original with him, but of course that did not interfere with the usefulness of the theory. I won't go into details about the method of preparing the degenerated pancreas, a source

VII

DIABETES AND THE INSULIN PROBLEM

By William C. Stadie

I shall discuss four topics relating to the action of insulin on intermediary metabolism in the mammalian organism. These are: 1) the action of insulin on glucose transport across cell boundaries; 2) some aspects of the metabolism of fat in the diabetic; 3) the possible impairment of oxidative phosphorylation in the diabetic state; and 4) some data on the Houssay phenomenon manifested *in vitro*.

In the diabetic, an early defect in glucose metabolism was long predicated, namely, impairment of the glucohexokinase reaction yielding the first glucose metabolic intermediary. A step antecedent to this, namely, transport of glucose across cell membranes to sites of enzymatic action, received scant attention. Hoeber and Loewi discussed this possibility but presented no supporting evidence. Levine and his group[1,2,3] were the first to publish strong evidence in support of the hypothesis. To determine whether insulin increased the rate of entry of sugars into the tissue, they selected galactose because it is essentially inert in the peripheral tissues. Eviscerated animals were used to limit the metabolism of galactose to the periphery. The type of experiment they reported is illustrated in Figure 1.

Galactose without and with insulin was injected intravenously and galactose blood levels determined over a period of four to six hours. Constant blood levels were attained in two to four hours, from which were calculated the galactose volume of distribution. Without insulin, this was approximately 45% of the body weight and with insulin, it was increased to approximately 70%, essentially that of body water. They divided the sugars they tested into two classes: 1) sugars nonresponsive to insulin; and 2) sugars responsive to insulin. The volumes of distribution of the latter were increased by [illegible] Th[illegible]e [illegible]thors noted that the ins[illegible], D-xylose and D-

as we got from normal beef pancreas. This is the material that I had hoped would be first used clinically, because it was so easy to prepare and so potent. We put it through a Berkfeld filter and it came through without any loss of potency. Banting objected because "they'll say we didn't make the first extract for human beings from a commercially available source." Actually, we never had any time to prepare or fractionate our material for clinical use. We were precipitated into the clinical trials. We never had an opportunity to fractionate our product, as we should have liked, before insulin was given to the first case. Our first human trial was quite successful. The extract was potent, but not nearly as potent, nor as pure, as the material from the fetal calf pancreas. Recently, we have made a lot from fetal pancreas, using exactly the original procedure. We obtain a fine yield—8 to 10 units per gm. The extract looks good, and I'm going to persuade some of the clinicians to try it. The method, acid alcohol extraction, taking off the alcohol, and taking the material down to dryness, then washing thoroughly with fat solvents, and with 95% alcohol gives a nice product, particularly with fetal pancreas. It can, I would think, be used clinically without any difficulty.

The other points, which we tried to establish, were decreases in ketone body excretion, and diminution in nitrogen excretion. We found a gain in weight in our animals, and recorded that they appeared to be fatter. A little later, in December of 1921, when Professor Macleod and Professor Collip actively joined the group, Dr. Macleod really took over the planning. Glycogen formation and decrease in the fat content of the liver were demonstrated. I chose as my problem the effect of insulin on the respiratory quotient, and wrote my M.A. thesis in physiology that year on the very dramatic rise we got in dogs, not with sugar alone but with sugar plus insulin. There was a dramatic rise in Joe Gilchrist's R/Q. He was our first doctor patient.

The journal *Diabetes* is printing some special articles about the early work on insulin in their next issue to mark the thirty-fifth Anniversary of the discovery.

I have already apologized to Dr. Stadie for introducing this background material, and now for the next 45 minutes, if you like, Dr. Stadie, we would like to hear about your work.

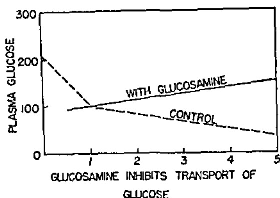


FIG. 2. The figure shows that when D-glucosamine hydrochloride is injected

The rabbits were simultaneously injected with glucose and glucosamine. The data show that the plasma glucose levels, when glucosamine is simultaneously injected, are maintained at approximately the same level in contrast to the results obtained in the absence of glucosamine when there is the expected fall in plasma glucose.

Park, Bornstein, and Post[12] were the first to obtain direct evidence that the transport of glucose across cell membranes was accelerated by insulin. Using isotopic glucose and the rat diaphragm, they devised methods to determine intercellular free glucose. Intercellular free glucose could be influenced in two ways: 1) acceleration of transport by insulin would increase intercellular

FIG 3. EFFECT OF INSULIN ON FREE GLUCOSE IN RAT DIAPHRAGM:
t=37°, GLUCOSE=2%, S A.=19,400
Free Glucose (N=3)

Ins.	*Total (CPM)*	*S.A. (CPM/MG)*
0	84,000	19,200
+	125,000	19,600

FIG 3 Data taken from Park, C R, and Johnson, L. H.: *Am. J. Physiol.*, *182*.17, 1955

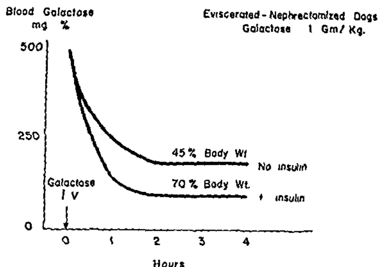
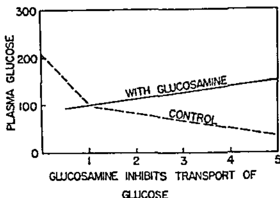


FIG. 1. Galactose was injected at 0 time. Note that insulin caused a faster and wider distribution of the galactose. The galactose "space" in the absence of added insulin was about 45% of body weight; in the presence of added insulin this space widened to about 70% of body weight. (Data taken from: Levine, R., Goldstein, M. S., Huddlestun, B., and Klein, S.: *Am. J. Physiol.*, *163*:70, 1950)

galactose, are all characterized by having the same configuration in carbons 1, 2, and 3. From these data they concluded that the entrance of sugar into muscle "is governed by a transfer system which is specifically adapted with respect to chemical structure." Since glucose resembles those responsive sugars structurally, they concluded that glucose is also insulin responsive.

These conclusions of Levine and his group received support from a number of workers. Ross,[4,5,6] studying the rate of passage of glucose from blood to aqueous humor of the eye across the cell boundary of the ciliary body, showed that the rate of transfer was significantly diminished in the alloxanized diabetic rabbit. Drury and Wick[7,8,9] reported experiments which confirmed the concepts of Levine. They reported that mannose[10] and glucosamine[11] were also insulin responsive. They also showed that insulin responsive sugars competed with each other when both were simultaneously injected into the experimental animal. Illustration of this type of competition is shown in Figure 2.



GLUCOSAMINE INHIBITS TRANSPORT OF GLUCOSE

(Data taken from. Wick, A N., Drury, D R., Nakada, H. I, Barnet, H. N., and Morita, T. N.: *J. Biol. Chem*, *213*.907, 1955.)

The rabbits were simultaneously injected with glucose and glucosamine. The data show that the plasma glucose levels, when glucosamine is simultaneously injected, are maintained at approximately the same level in contrast to the results obtained in the absence of glucosamine when there is the expected fall in plasma glucose.

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0	84,000	19,200
+	125,000	19,600

FIG. 3. Data taken from Park, C R, and Johnson, L H · *Am J Physiol*, *182* 17, 1955.

free glucose; or 2) acceleration of the hexokinase reaction would decrease it. Park used two devices to study these possibilities: 1) high concentrations of glucose in the medium, namely, 2% at 37°; or 2) lowering the temperature of equilibration to 15°C. Under these circumstances he showed, as Figure 3 illustrates, that insulin in the medium increased the amount of free glucose within the diaphragm. It is also to be noted that the specific activity of the free glucose was essentially that of the medium glucose.

FIG. 4. INSULIN DOES NOT ACCELERATE TRANSPORT OF GALACTOSE INTO BRAIN

	Ratio Tissue to Serum Galactose	
	Insulin	
	0	+
Diaphragm	0 10	40
Heart	0 13	.70
Gast-Nem	0 07	.4
Brain	0.56	0.57

FIG. 4. Data taken from: Park, C. R., and Johnson, L. H.: *Am. J. Physiol., 182*:17, 1955.

In other experiments, Park *et al.* showed that the specific activity of the free glucose increased with time, ultimately reaching the level of medium glucose. Park and his colleagues[13] reported a variety of experiments of this same character. In Figure 4 is shown the ratio of tissue to serum galactose with and without simultaneous injection of insulin. An increase of this ratio is assumed to indicate an action of insulin in accelerating transport. The diaphragm, the heart, and the gastrocnemius muscle show by this criterion that galactose is insulin responsive. In the brain, however, insulin had no effect in accelerating the transport of galactose across the blood-brain barrier. This observation should be associated: 1) with the general conclusion in the literature that no action of insulin upon brain metabolism has been established; and 2) with the observation Haugaard and I[14] reported using isotopic insulin, that brain was the only tissue in which no insulin was found following intravenous injection. The summation of

this evidence, particularly the observation that insulin increased rather than decreased the intercellular free glucose, brought Park and his coworkers to the conclusion that the action of insulin on glucose uptake of diaphragm is concerned with the mechanism of transfer of glucose into the tissues and that this transfer acceleration antecedes and is distinct from glucose phosphorylation by the hexokinase reaction.

The ascendency of the transport hypothesis stimulated our laboratory to restudy a phenomenon which Zapp and I originally reported 13 years ago. We observed that under certain circumstances the aerobic production of lactic acid from glucose by the rat diaphragm was large and not influenced by insulin.

FIG 5 CONVERSION BY RAT DIAPHRAGM OF U-C^{14}-GLUCOSE TO LACTIC ACID (uM/GM/2Hr.)

Rat No		*From Glucose*		*% From Glucose*	
	Ins	0	+	0	+
7		73	73	109	106
8		123	125	72	74
9		142	128	61	59
10		137	132	58	57
11		168	155	57	57
12		153	108	68	65
46		108	105	78	76
47		83	45	66	50
	Mean ± Sem	123 ± 8	109 ± 13	71 ± 6	68 ± 6

FIG 5. Data taken from: Shaw, W. N , and Stadie, W. C.: Unpublished Experiments, 1956.

These same diaphragms, however, uniformly responded by synthesizing extra glycogen from glucose when insulin was present in the medium. In Figure 5 is shown some data recently obtained by Shaw in our laboratory. Using isotopic glucose in the medium, the effect of insulin upon the glycolytic intermediaries was studied. Figure 5 shows individual and mean values of lactic acid production. As in our older observations, the total lactic acid is uninfluenced by insulin; in addition, that derived from the medium glucose, which accounts for approximately ¾ of the lactic acid, is also uninfluenced by insulin.

FIG. 6. CONVERSION BY RAT DIAPHRAGM OF U-C^{14}-GLUCOSE TO GLYCOGEN INSULIN EFFECT, uM/GM/2 HR.

Rat No.		*On Total Glycogen*	*On Glycogen From Gluc.*	*Ratio Col. 2/1*
7		14	19	1.32
8		50	19	0.38
9		13	7	0 55
10		17	13	0.77
46		13	7	0.52
47		19	14	0.74
	Mean	21	13	0 71
	Sem	±6	±2	0.15

FIG. 6. Data taken from: Shaw, W. N., and Stadie, W. C.: Unpublished Experiments, 1956.

In contrast, we see in Figure 6 that these same diaphragms show an insulin effect upon total glycogen synthesis. The last column shows that approximately ¾ of the insulin effect upon glycogen synthesis is accountable by conversion of medium glucose to glycogen. It is difficult to explain these effects in terms of the conventional Embden-Meyerhof scheme of glycolysis for it would be expected that acceleration of glucose to the branch point of glucose-6-phosphate should be associated with an increase of lactate formation as well as glycogen.

We studied the problem further by isolating from the diaphragm the intermediary phosphate esters, using appropriate chromatographic methods.

FIG 7. GLUCOSE-6-PHOSPHATE ISOLATED FROM RAT DIAPHRAGM AFTER EQUILIBRIUM WITH U-C^{14}-GLUCOSE ($N=7$)

	Total uM/Gm/2 Hr.		*% from Glucose*	
	0	+	0	+
Mean	.30	38	46	83
Sem	.14	12	9	10
Diff.				37±14
t				2 7

FIG. 7. Data taken from· Shaw, W N., and Stadie, W C.: Unpublished Experiments, 1956.

Figure 7 gives data on glucose-6-phosphate isolated from the diaphragm after 2-hour equilibration with uniformly labeled glucose. The mean results indicate clearly that there is a more rapid turnover, approximately double, of glucose-6-phosphate when insulin is present in the medium.

FIG. 8. CONVERSION OF U-C^{14}-GLUCOSE TO G-1-P IN NORMAL RAT DIAPHRAGM MEAN ($N=8$)

	Total uM/GM/2 Hr	*% From Glucose*
+ Insulin	4 6	78
0 Insulin	2.6	45
Difference	2 0*	33

* Significant.

FIG. 8 Data taken from: Shaw, W. N., and Stadie, W. C.: Unpublished Experiments, 1956.

Figure 8 shows results obtained in similar fashion on the isolated glucose-1-phosphate. Again in the presence of insulin the turnover of glucose-1-phosphate from medium glucose is approximately double. You will recall that lactate recovered from the medium showed specific activities which were essentially the same in the presence or absence of insulin. Lactate, therefore, could not have been derived from the glucose-6-phosphate pool which we recovered from the diaphragm. This apparently paradoxical situation was explained by the findings in subsequent experiments.

Figure 9 shows data on fructose-1-6-diphosphate isolated from these diaphragms. In no case have we been able to demonstrate

FIG 9 FRUCTOSE-1-6-DIPHOSPHATE ISOLATED FROM NORMAL RAT DIAPHRAGM AFTER EQUILIBRIUM WITH U-C^{14}-GLUCOSE

$N=8$ Mean Values uM/GM/2 Hr

	Total		*From U-C^{14}-Glucose*	
	0	+	0	+
Insulin	45	51	02	02

FIG 9. Data taken from: Shaw, W. N., and Stadie, W. C: Unpublished Experiments, 1956.

that any significant quantity of fructose-1-6-diphosphate recovered from the diaphragm, i.e., "internal" F-1-6-P, was derived from medium glucose.

The possibility of a pathway of lactate formation from glucose other than through the Embden-Meyerhof scheme was ruled out by experiments of a type illustrated in Figure 10.

FIG. 10. FORMATION (RAT DIAPHRAGM) OF LACTIC ACID FROM U-C^{14}-GLUCOSE IN PRESENCE OF NONLABELED METABOLITES

Added	*Per Cent of Lactic Acid From Glucose*	
	Control	*Test*
Gluconate	53	49
6-P-Gluconate	92	91
Pyruvate	103	36
Glucose-1-P	89	84
Glucose-6-P	82	70
Fructose-1-6-P	81	49
3-P-Glycerate	79	47

FIG. 10. Data taken from: Shaw, W. N., and Stadie, W. C.: Unpublished Experiments, 1956.

Certain nonlabeled metabolites were included in the medium in addition to C^{14}-glucose. Neither gluconate nor 6-phosphogluconate diluted the isotopic lactate—neither did glucose-1-phosphate. In contrast, pyruvate, fructose-1-6-diphosphate and 3-phosphate glycerate produced significant dilution of the lactate derived from isotopic glucose. This situation, which at the moment seemed confusing became clarified when we studied what happened when a small amount of isotopic glucose-6-phosphate was added to the medium in the presence of nonisotopic glucose.

The data in Figure 11 shows that as usual there is no insulin effect upon lactate production but a significant amount, approximately 10%, was derived from the isotopic glucose-6-phosphate. In contrast, none of the label originally present in the glucose-6-phosphate was recovered from the glycogen although the insulin effect was manifest as usual. The conclusion is that glucose-6-phosphate in the medium enters readily into the enzymatic system produc-

FIG 11. RAT DIAPHRAGM: CONVERSION OF ISOTOPIC G6P IN MEDIUM TO LACTATE AND GLYCOGEN IN PRESENCE OF NONISOTOPIC GLUCOSE:

(*Mean uM/GM/2 Hr; N=6*)

	Lactate		*Glycogen*	
	Total	*From G6P*	*Total*	*From G6P*
No Insulin	118	11	30	0
Insulin	101	10	40	0

FIG. 11. Data taken from· Shaw, W. N., and Stadie, W. C.: Unpublished Experiments, 1956.

ing lactate but it is completely barred from the system producing glycogen.

Figure 12 shows a similar type of experiment. In this case, non-isotopic phosphate esters were added to the medium and after equilibration for two hours in the presence of isotopic glucose, the esters were reisolated from the medium, quantitated and counted, and compared with the values obtained from the lactate. In the case of glucose-6-phosphate and fructose-1-6-diphosphate, the specific activities are essentially those of the lactate, indicating that these esters freely interchange with isotopic glucose and lactate. As before, glucose-1-phosphate in the medium has no effect upon the specific activity of the lactate derived from the glucose, and when reisolated it was found completely devoid of radioactivity.

FIG. 12. INSULIN NONRESPONSIVE GLYCOLYSIS. INTERCHANGE OF NONLABELED ESTERS IN MEDIUM WITH ISOTOPIC GLUCOSE

Ester in Medium	*Lactate from Gluc*		*Reisolated Ester*
	%	001 S A.	.001 S A.
G-6-P	70	17	16
F-1-6-P	49	14	16
G-1-P	84	23	0
Medium Glucose		20	

FIG. 12. Data taken from: Shaw, W N., and Stadie, W C. Unpublished Experiments, 1956

FIG. 13. "INTERNAL" AND "EXTERNAL" FRUCTOSE-1-6-DIPHOSPHATE FORMED FROM U-C^{14}-GLUCOSE BY RAT DIAPHRAGM

	.001 S.A. CPM Per C_6	
	No Ins.	*Ins.*
F-1-6-P From		
Medium	10.2	10.6
Diaphragm	0	0
Lactate	8.7	6.5
Glycogen	2.8	5.1
Glucose in Medium	10.2	

FIG. 13. Data taken from: Shaw, W. N., and Stadie, W. C.: Unpublished Experiments, 1956

Figure 13 shows some interesting data obtained when nonisotopic fructose-1-6-phosphate was present in the medium together with isotopic glucose. At the end of the equilibration period, fructose-1-6-phosphate was isolated both from the medium and the diaphragm. The data give a specific activity in cpm/glucose equivalents. Note that the customary insulin effect upon glycogen was obtained whereas there was none upon lactate formation. The fructose-1-6-diphosphate derived from the medium had essentially the same specific activity as that of the lactate; there was no difference effected by presence of insulin. On the other hand, the fructose-1-6-diphosphate isolated from the diaphragm had none of the label.

To recapitulate: The data suggest that there are two coexisting glycolytic systems in the diaphragm. These appear to be of the Embden-Meyerhof type but are different in two respects: The system concerned with the production of glycogen from medium glucose is insulin responsive. It synthesizes extra glycogen from the glucose and the glucose-6-phosphate and the glucose-1-phosphate isolated from the diaphragm show accelerated turnover rates. The second enzymatic system is mainly concerned with the production of lactic acid. In contrast to the first system it is nonresponsive to insulin. Another difference is apparent. The second system has no barrier preventing the ready exchange of phos-

phorylated intermediaries from the medium to the enzyme system itself. In contrast, phosphorylated intermediaries in the medium do not interchange with the first system.

These observations appear to us to be reasonably explained by the concept of Levine that there exist in tissues barriers two sites of enzymatic action which oppose the ready entrance of metabolites, and that these barriers are influenced by a hormone. The system concerned with glycogen synthesis possesses a barrier to glucose which is insulin responsive. In addition, the barrier prevents the entrance and exit of phosphorylated intermediaries. This explanation appears to be more reasonable to us than the alternative one, namely, to suppose that two different hexokinase systems exist in the diaphragm, one of which is responsive to insulin and the other not.

THE RELATION OF INSULIN TO FATTY ACID METABOLISM

The relation of insulin to fat metabolism is still obscure. This is not surprising since the intermediary metabolic pathways of fat metabolism have only recently been clarified. Lipogenesis both in the intact animal and in preparations studied *in vitro* is depressed in certain nutritional states. These are: fasting; or drastic reduction in carbohydrate intake. Fasting for as little as 12 hours produces a significant decrease in the incorporation of acetate into fatty acid by liver slices. Conversely, the decrease of lipogenesis from acetate following fasting can be completely reversed in a few hours by an injection of glucose. In the experimental diabetic animal, lipogenesis is seriously impaired, a phenomenon first demonstrated by the experiments of Stetten and Klein[15,16] who studied the incorporation of deuterium into liver and carcass fats. The impaired lipogenesis observed in the intact diabetic animal can also be demonstrated using liver slices *in vitro*. This impairment of fatty acid synthesis is reversed by treatment of the intact animal with insulin. The reversal requires an appreciable time and maximal restoration is reached in approximately 16 to 24 hours. In contrast, the reversal of impaired lipogenesis in liver slices by the addition of insulin to the medium has not been accomplished. This is in contrast to the situation in the diaphragms

from normal or diabetic animals which respond readily to insulin in the medium so that glycogen storage and glucose uptake is restored immediately to essentially normal levels.

Much of the experimentation and discussion centers around the possibility that a second metabolic block exists in the diabetic affecting adversely the complete oxidation or the synthesis of fatty acids. It is possible that part of the impairment of fatty acid metabolism is secondary to impairment of glucose metabolism. But there is evidence which points to a defect in fat metabolism per se. I shall present some evidence which supports this view.

FIG 14. LIVER SLICES FROM DIABETIC RATS

Nutrilite	CO_2	Synth. Fatty Acids
Glucose	0	0
Fructose	Normal	0
Acetate	Normal	0

FIG. 14. Data taken from: Chernick, S. S., and Chaikoff, I. L.: *J. Biol. Chem.*, *188*:389, 1951.

In Figure 14 are data from experiments of Chaikoff[17] using liver slices from diabetic rats in the presence of three nutrilites: 1) glucose; 2) fructose; and 3) acetate. Isotopic carbon incorporated to CO_2 and higher fatty acids was measured after an appropriate period of equilibration. The data of the figure show that glucose was essentially inert as would be expected. Fructose which is insulin nonresponsive with respect to transport is readily phosphorylated by the diabetic liver slice and CO_2 formation was essentially normal. But no incorporation of fructose into fatty acids was observed. The results with acetate were similar to those with fructose.

Figure 15 shows further data of Chaikoff.[18] Diabetic liver slices were equilibrated with isotopic lactate with C^{14} in the 1, 2, or 3 position respectively. Following equilibration, the recovery of the isotopic carbon from the higher fatty acids was essentially 0. Liver slices from insulin treated diabetic rats incorporated both carbon-2 and carbon-3 of lactate into higher fatty acids. In the diabetic slice, recovery of the carboxyl carbon in the CO_2 is essentially normal. This is a reasonable index of the formation of C-2 inter-

FIG. 15 DIABETIC RAT LIVER SLICES

CO_2 and Fatty Acid Synthesis from 1-C^{14}, 2-C^{14}, or 3-C^{14} Lactate

Per Cent C^{14} Recovered	
From CO_2	From Fatty Acids
Normal	± 0

FIG. 15. Data taken from: Felts, J. M., Chaikoff, I. L., and Osborn, M. L.: *J. Biol. Chem.*, *191*.683, 1951.

mediaries. It, therefore, can be concluded that acetyl CoA formation in the diabetic liver slice is essentially normal. Apparently then the pathway from acetyl CoA to fatty acid is impaired in the diabetic. Further studies by Chaikoff,[19] using pyruvate-2-C^{14} gave essentially the same results. In general, treatment of the intact rat with insulin for one or two days restores the ability of the diabetic liver slice to incorporate pyruvate, lactate, or acetate into higher fatty acid. From the summation of this evidence, Chaikoff concluded "that insulin is involved either directly or indirectly at more than one point in metabolic pathways in the liver. The entrance of lactate into the cycle is not blocked in the diabetic liver. Insulin does, however, direct the fate of the C-2 fragment from an oxidative pathway to one involving fatty acid synthesis."

Gurin and his group[20] pushed this aspect of fatty acid metabolism in diabetes further by studying the conditions under which hepatic mitochondria incorporated acetate, or pyruvate into higher fatty acids. They prepared two fractions from liver homogenates: 1) washed mitochondria by conventional techniques; and 2) a supernatant fraction centrifuged at 100,000 G for one hour to remove all particulate matter. In brief, mitochondria plus supernate from normal rat livers incorporated acetate, lactate, or pyruvate into fatty acids. In contrast, mitochondria plus the supernatant fraction from the livers of diabetic rats or pigeons did not do so. To eliminate difficulties introduced by permeability factors, Shaw and Gurin prepared extracts of mitochondria and studied their ability to incorporate various substrates into fatty acids. The system consists of mitochondrial extract plus supernatant fraction, together with Mg, citrate, and diphosphopyridinenucleotide.

FIG 16. FATTY ACID SYNTHESIS BY RAT LIVER FRACTIONS:
System: Mitochondrial Extract, Supernatant Fraction, Mg Citrate, DPN

Added	*Fatty and Synth.*	
	Normal	*Diab.*
Acetate	Yes	No
Acetyl CoA	Yes	No
Pyruvate	Yes	No
Pyr. or Acet.+N. Super.		Yes
Acetate +F-1-6-P		Yes
Acetyl CoA +F-1-6-P		Yes
Pyruvate +F-1-6-P		Yes

FIG. 16. Data taken from: Shaw, W. N., and Gurin, S.. Unpublished Experiments, 1956.

The data in Figure 16 show that mitochondrial extract plus supernate prepared from normal livers readily incorporates acetate, acetyl CoA, or pyruvate into higher fatty acids. A similar system prepared from the diabetic animal does not. In the diabetic, the further addition of normal supernatant restores the synthesis. Incorporation of acetate, acetyl-CoA, or pyruvate is also achieved when fructose-1-6-diphosphate is added to the diabetic system. The conclusions from this experiment drawn by Shaw and Gurin are tentative. In some manner not yet clear Fructose-1-6-diphosphate bridges the gap between acetate or acetyl CoA to higher fatty acids. Apparently, this is not due to glycolysis of the fructose-1-6-diphosphate to pyruvate since pyruvate itself does not restore fatty acid synthesis.

Figure 17 from date of Shaw and Gurin[21] suggests a possible localization of the diabetic defect which prevents the incorporation of pyruvate into fatty acid by mitochondrial preparation. The system consists of whole mitochondria without the supernatant fraction, together with Mg, citrate, DPN, and isotopic pyruvate plus butyryl CoA. Such a system incorporates pyruvate into fatty acid, both in the normal and the diabetic instances. If the butyryl CoA is omitted from the system, fatty acid synthesis does not occur. From these experiments, Shaw draws the conclusion: "Butyryl CoA appears to be able to replace the supernatant fraction of both

FIG. 17. FATTY ACID SYNTHESIS BY WHOLE RAT LIVER MITOCHONDRIA

Complete System: Whole Mitochondria, Mg, Citrate, DPN, Isotopic Pyruvate, Butyrl CoA (No Supernatant Fraction)

System	Norm.	Diab
Complete	Yes	Yes
Without Butyryl CoA	No	No

FIG. 17. Data taken from: Shaw, W. N, and Gurin, S.: Unpublished Experiments, 1956.

the normal and diabetic water-soluble system. It seems reasonable, therefore, to suggest that the diabetic liver is unable to convert pyruvate, acetate, or acetyl CoA to fatty acids because it cannot convert acetyl-CoA to butyryl-CoA. This conversion involves two oxido-reductive steps which in fatty acid synthesis are: 1) the reduction of acetoacetyl-CoA to B-hydroxybutyryl CoA; and 2) the hydrogenation of B-hydroxy-butyryl-CoA to butyryl-CoA. The former step requires reduced DPN; the latter, reduced FAD. This failure in the diabetic liver which appears in the supernatant fraction of the liver homogenate may be due to the inability of the soluble enzyme systems to supply one or both of these two cofactors."

FIG 18. KETONE FORMATION BY LIVER SLICES OF TREATED DEPANCREATIZED CATS

Cat No.	*Ins Units*	*Time of Treatment Hr*	*Ketones Formed uM/GM/Hr.*
1	6	1 5	53
2	40	6	17
3	35	6	17
4	30	5	18
Mean No Treatment			50

FIG. 18. Data taken from: Stadie, W C., Zapp, J. A., Jr., and Lukens, F. D. W: *J. Biol. Chem., 132*.432, 1940

Figure 18 shows some data from our laboratory bearing on the relationship of insulin to fatty acid metabolism.[22] The animal was

the depancreatized cat, used 48 to 72 hours after operation. They were characterized by 4 plus ketonuria and a very marked ketonemia. Liver slices from such animals equilibrated in a phosphate saline medium respire at a constant rate up to six hours, and produce at an essentially unaltered rate large amounts of ketone bodies. It is possible to diminish significantly the rate of formation of ketones by the liver slice by prior treatment of the cat with insulin. Data showing this are shown in Figure 18. Note that a short treatment of one to two hours is without effect and that significant decrease in ketone formation requires vigorous treatment for a period of five to six hours. Such treatment reduces the ketone formation to approximately ⅓ of that observed in the completely diabetic state.

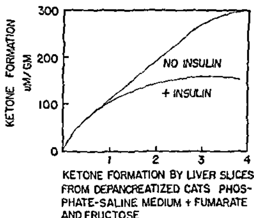


FIG. 19. Data taken from: Stadie, W. C., Zapp, J. A., Jr., and Lukens, F. E. W.: *J. Biol Chem.*, *132*:432, 1940

We were partially successful in demonstrating an action of insulin *in vitro* in decreasing ketone formation by diabetic cat liver slices. In the type of experiment shown in Figure 19, the slices were equilibrated for four hours, the medium being changed every hour. Our best results were obtained when the medium contained fructose + fumarate. The curves show total ketone formation with these nutrilites without and with insulin. In the absence of insulin, there is no great change in the rate of formation of the ketone bodies. In the presence of added insulin, however, ketone formation falls off to low values at the end of four hours. Fructose

per se or its glycolytic products or the reinforcement of Krebs' intermediaries appear to be inadequate to change the diabetic type of fat metabolism in these slices. Apparently, insulin supplements this system so that fat metabolism proceeds in some direction other than ketone formation. Two possibilities are obvious: 1) that fatty acid metabolism is now directed toward complete oxidation to CO_2 rather than ketone formation; or 2) the excessive oxidation of fat characteristic of the diabetic liver metabolism ceases, and some other type of metabolism takes up the function of energy production for hepatic metabolism.

We have been unable to reproduce this phenomenon constantly. About 1/3 to 1/2 of our attempts fail for reasons not clear.

These experiments are reminiscent of the report of Hastings and his group on "Biochemical Sequence of Events after Insulin Administration."[23] They reported that insulin exerts immediate metabolic effects upon the peripheral tissue as represented by rat diaphragm. In contrast, prior treatment of the intact diabetic rat with insulin for 6-24 hours is required to produce significant restoration toward normal of glucose metabolism by the liver slices *in vitro*. They called attention to the fact that no demonstration of insulin action *in vitro* on diabetic liver slices has been reported. These considerations prompted Hastings' group to discuss a possible role of insulin in "adaptation" of enzyme systems in the liver.

OXIDATIVE PHOSPHORYLATION IN THE DIABETIC ANIMAL

My third topic is the problem of oxidative phosphorylation in the diabetic animal. The relation of phosphate metabolism to the diabetic state has received a considerable amount of attention in the diabetes literature. A defect in oxidative phosphorylation decreasing available high energy phosphate, e.g., adenosinetriphosphate or other energy rich bonds, might explain some of the observed metabolic defects in the diabetic However, few authorities espouse the hypothesis and the evidence for it is scanty and indirect. I shall cite some of the most pertinent evidence in the literature, together with some recent experiments from our laboratory.

Kaplan[24,25,26,27] and his coworkers reported much experimental evidence on the incorporation of radioactive phosphate into various organic phosphate compounds of liver and muscle 1-2 hours after injection. In general, they concluded that insulin brings about an increased turnover rate of isotopic phosphate in these organic fractions. Sacks[28] concerned himself with experiments of this type, one of which I show in Figure 20.

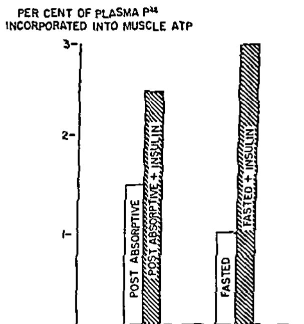


FIG. 20. Effect of insulin upon oxidative phosphorylation: The experiment shows the rate of incorporation of inorganic phosphate of the blood into ATP of cat muscle after injection of P^{32} and insulin. Note that insulin increased the rate of turnover of P^{32} in the ATP (Data taken from. Sacks, J.: Cold Spring Harbor Symp. *Quant. Biol.*, *13*:180, 1948)

The data show that in both the fasting state or following glucose administration isotopic phosphate is incorporated more rapidly into adenosinetriphosphate of muscles in cats following the simultaneous injection of insulin. Sacks[28] reported many other experiments dealing with this general question; his general conclusion is that the administration of insulin results in an increase of phosphate turnover in muscle due to an acceleration of oxidative phosphorylation.

Goranson, Hamilton, and Haist[30] reported experiments leading to conclusions of the same general nature.

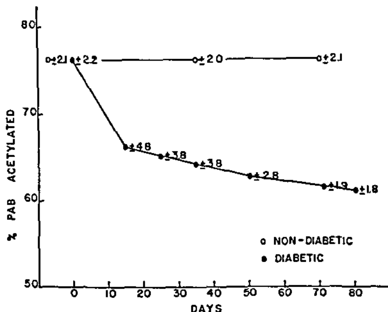


FIG 21. Data taken from. Charalampous, F. C., and Hegsted, D. M.: *J. Biol. Chem., 180* 623, 1949. The curves show that the acetylation of injected para-aminobenzoic acid in the diabetic rat is impaired, compared to the normals. This is presumably due to diminished oxidative phosphorylation.

Charalampous and Hegsted[31] reported experiments shown in Figure 21. The acetylation of aromatic amines is dependent upon the availability of energy rich phosphate bonds. Charalampous, therefore, studied this reaction in the intact normal and alloxan diabetic rat following the injection of paraaminobenzoic acid. Percentage of PAB acetylation was taken as a measure of the availability of adenosinetriphosphate. As the data in Figure 21 show, there was a highly significant fall in acetylation in the diabetic animal which persisted throughout the course for as long as 80 days. This decreased acetylation was restored to normal by the injection of insulin. The authors concluded "that it is reasonable to assume that a primary deficiency of ATP is the cause of a metabolic defect."

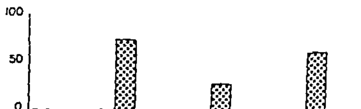


FIG. 22. There is diminished phosphorylation of thiamine in the alloxan diabetic rat, compared to the values obtained in the normal. This is presumed due to deficient oxidative phosphorylation. (Data taken from: Foa, P. P., Weinstein, H R, Smith, J. A., and Greenberg, M.: *Arch Biochem.*, *40*:323, 1952.

In Figure 22 Foa[32] and his coworkers report data on another ATP dependent reaction, namely, the phosphorylation of thiamine. In livers of the alloxan diabetic rat, there is no decrease of the total thiamine. However, the percent of thiamine which is phosphorylated is significantly lower than in the normal, viz., a value of 34 as contrasted to 82. They also reported that insulin restored toward normal the thiamine phosphate content of the alloxan diabetic livers.

We have been long interested in this area of diabetic metabolism and recently Vester,[33] in our laboratory, has approached the problem directly by determining the rate of oxidative phosphorylation by mitochondria prepared from livers of depancreatized cats. The mitochondria were prepared in the conventional fashion and equilibrated at 30° with excess hexokinase and glucose, using as nutrilite either pyruvate or beta hydroxybutyrate. Glucose-6-phosphate formation was equated to ATP synthesis as a measure of oxidative phosphorylation. The data in Figures 23 and 24 are expressed in two forms: 1) micromoles of ATP formation per gram of mitochondrial protein per minute; and 2) as P:O ratios. In our first series of nine depancreatized cats, used 48-72 hours after opera-

FIG. 23. OXIDATIVE PHOSPHORYLATION BY MITOCHONDRIA FROM LIVERS OF NORMAL AND DEPANCREATIZED CATS

	N	*Substrate*	*ATP Synthesis*	*P:O*
Normal	6	Pyruvate	58±2.9	1.88±.29
Depan.	5		26±9.2	89±.20
	Diff.		32±9.6	.99±.35
	t		3.3	2.6
Normal	6	β-OH Butyrate	36±3.3	1.71±.11
Depan.	5		22±6.4	.78±.20
	Diff.		14±7.1	.93±.23
	t		2 0	4.1

FIG. 23. Data taken from: Vester, J. W, and Stadie, W. C.: Unpublished experiments, 1956

FIG. 24 OXIDATIVE PHOSPHORYLATION BY MITOCHONDRIA FROM BIOPSIES OF LIVERS OF DEPANCREATIZED CATS

Cat No.	*ATP Synthesis uM/GM PROT./MIN Status*		*P:O Status*	
	Diabetic	*Treated*	*Diabetic*	*Treated*
I	—	200	—	3 0
	—	135	—	2.3
	—	110	—	2 6
IV	—	170	—	2 9
	107	—	2 4	—
V	50	68	1 8	2 5
	98	168	2 6	3 0
VIII	12	138	0 7	3 0
IX	93	89	1 9	2 3
X	—	117	—	2.9
XI	—	120	—	3 1
	94	—	2 1	—
Means	76	132	1.8	2 8
Sem	14	12	4	0 1
Difference		56±19		1 0±.4
t		3 0		2.5

FIG 24 Data taken from. Vester, J W., and Stadie, W. C: Unpublished experiments, 1956.

tion, we studied only the oxidative phosphorylation capacity of isolated mitochondria, using the two nutrilites listed, namely, pyruvate and beta hydroxybutyrate. All the data are included in the figure and, as is shown by the values of the means with their standard errors, the rate of oxidative phosphorylation per *unit of mitochondrial* protein is significantly different in the diabetic cat with both nutrilites compared to the normal. The same holds true for the P:O ratios. The data appear to lead to the conclusion that the livers from depancreatized cats show an impaired oxidative phosphorylation.

In these *in vitro* experiments, addition of insulin gave results no different from those obtained in the absence of insulin. We, therefore, attempted to demonstrate an insulin reversal by treatment of the depancreatized animal with insulin. Our data were obtained in the following way: After pancreatectomy, the animals were permitted to develop a completely diabetic status as shown by glycosuria and marked ketonuria (periods up to 72 hours). They then were treated with insulin for periods up to three to four days in an attempt to restore them to a normal status. Glycosuria and ketonuria were never completely eliminated but, in contrast to the diabetic status, they were markedly reduced. Their clinical state was much improved and they ate about as much as normal cats. A liver biopsy was then done, sufficient liver being taken to yield mitochondria for assay. The cats were then permitted to relapse again into the diabetic status; another biopsy for assay was done. Restoration toward the normal status was again attempted. In some animals, these cycles were repeated as many as 4 times, with some blanks which could not be filled in owing to the bad clinical conditions of the cats.

If we follow the data through the cycles in each individual cat, the general conclusion may be drawn, namely, that in the diabetic status both the total oxidative phosphorylation and the P:O ratios were less than those observed in the restored state. When the data are summarized as means of the values in the diabetic and normal status, it will be seen, as in our first series, that there is a significant diminution of oxidative phosphorylation in the "diabetic status" in these animals and the same holds true of P:O ratio. What do these experiments mean in terms of the relation of

insulin to oxidative phosphorylation? We are not prepared to say until we are able to demonstrate an effect of insulin upon this phosphorylative activity *in vitro*. This we have not accomplished but are still seeking to do it. It is quite possible that the impaired phosphorylation which appears to exist in these depancreatized cat livers is merely an expression of the abnormal metabolic status of the liver subsequent to disturbed carbohydrate metabolism and impaired fat metabolism. In other words, the changes may be non-specific. Further work is necessary to answer these questions.

THE HOUSSAY PHENOMENON IN VITRO

The last topic I shall discuss is the Houssay phenomenon *in vitro*.

FIG. 25

	Acetate Incorporated by Rat Liver Slices into Long Chain Fatty Acids *Brady, Lukens, and Gurin (1951)* *Mg/100 mg* *Mean*	*Ketone Formation by Cat Liver Slices* *Stadie, Zapp, and Lukens (1940)* × *Micromoles/Kg Ca* *Mean*
Normal Cats	2–8	240
Depancreatized Cats	0–0.7	1200
Houssay Cats	5	85

FIG 25 Restoration of fatty acid metabolism toward normal in the Houssay cat.

Figure 25 shows two examples of this. The first was reported by Gurin and his coworkers[34] and the second comes from our laboratory. Gurin studied fat synthesis as measured by the incorporation of isotopic acetate into the higher fatty acids by liver slices from normal cats, from depancreatized cats, and from Houssay cats. As expected, the fat synthesis in the depancreatized animal liver slices is markedly impaired as contrasted to the normal. The removal of the pituitary in addition to the pancreas, restores fatty acid synthesis essentially to normal. In the second example are

shown data on ketone formation by the liver slices *in vitro*. The depancreatized cat liver slice produces large quantities of ketone bodies, but the Houssay preparation produces essentially none.

Apparently the Houssay preparation represents a basic type of "normal metabolism" upon which are superimposed insulin and pituitary factors in a state of balance. Excess or deficiency of one or the other results in abnormalities of metabolism which are restored to normal by a reinstitution of balance. The Houssay preparation represents a balance in which both factors have been reduced to 0.

FIG. 26. PLASMA INSULIN CONCENTRATION OF NORMAL DEPANCREATIZED, AND HOUSSAY CATS MICROUNITS PER ML.

	N	*Insulin Conc.*	*Recov. of Added 1000 Microunits %*
Normal	7	120	80–100
Depan	7	0	10–20
Houassay	2	0	100

FIG. 26. Data taken from: Unpublished data through courtesy of Dr. John Vallance Owen, 1956.

Figure 26 shows data which might be helpful in discussion of this phenomenon. I show these unpublished data through the courtesy of Dr. Vallance-Owen working in Lukens' laboratory. Dr. Vallance-Owen[35,36] employs a method for the assay of the insulin content of the plasma using the glucose uptake by the rat diaphragm The method is sufficiently sensitive to measure amounts of insulin of the order of 10 microunits per ml. or less. Vallance-Owen's data show that in the normal cat the levels of insulin per ml. of plasma run somewhere in the neighborhood of 100 to 200 microunits per ml When a thousand microunits per ml. is added to normal cat plasma, it may be demonstrated to be present by the rat diaphragm technique and essentially 100% of the activity is so demonstrated. In the depancreatized cat, the amount of insulin activity measured by the method is essentially 0. However, when 1000 microunits of insulin is added per cc. to such plasma,

Vallance-Owen is able to demonstrate the presence of only a fraction of it by his method. In the Houssay cat, again no insulin is demonstrable in the plasma but essentially 100% of the added insulin is recovered.

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DR. STADIE: I remember the paper of Park, *et al.** Our first studies of oxidative phosphorylation in the diabetic were done on mitochondria prepared from the livers of alloxanized rats. In a considerable number of experiments, we were unable to find any depression whatever of the P:O ratio or the rate of oxidative phosphorylation, in complete accord with the experiments of Park, Adler, and Copenhaver. However, the possibility brought out in the discussion by Dr. Houssay yesterday, that alloxanized rats may not be completely insulin free, prompted us to repeat the experiments using livers from depancreatized cats. Also in answer to your question about ATP, our system did contain a catalytic amount of ATP, and sufficient hexokinase to take care of four or five times the maximum expected phosphorylation. In addition, we did sham operations, using about the same manipulations used in pancreatectomy. The results with these preparations were essentially those observed in normal cats.

DR. LARDY: In the first set of data the controls gave a P:O ratio of 1.8, and the depancreatized rats were depressed to somewhat less than 1. In a later study, the diabetics gave a P:O ratio of 1.8. Insulin raised their phosphorylation efficiency to an average of about 2.8. Does this represent an improvement of techniques as time went on?

DR. STADIE: We realize that there is a considerable scattering of our data; that is one of the reasons why we showed all of it for critical evaluation. On a statistical basis, there is a high degree of probability that the sampling error has been wiped out by the large number of observations. However, we recognize the fact that there are factors of which we are unaware which produce variations in our values, both in the normal and the diabetic preparations.

DR. STETTEN: In relation to the first part of your presentation, I believe it has been established that a considerable number of the enzymes involved in the Embden-Meyerhof sequence can be

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DISCUSSION

DR. LARDY: I would like to ask a few questions about the oxidative phosphorylation defect that Dr. Stadie reported in the mitochondria of depancreatized rats. Some years ago three of my students in sequence collected data on the phosphorylation efficiency of mitochondria in alloxanized rats. The results have been published by Park, Adler, and Copenhaver, and they could find no evidence for a decreased phosphorylation efficiency after the rat had recovered from the immediate cytotoxic effects of the alloxan. I am wondering whether Dr. Stadie's control cats were subjected to a sham operation. I'm wondering also whether adenosine triphosphate was added to the incubation mixture. I notice that he reported micromoles of adenosine triphosphate synthesized in a system where hexokinase was present. By micromoles of ATP synthesized, do you mean micromoles of phosphate taken up?

nicely, and ketosis is inhibited. Insulin *in vitro* does not repair the disturbed lipogenesis. Would you then say that the defect in lipogenesis is due to the presence of the pituitary rather than to the absence of insulin?

DR. STADIE: Dr. Levine, I believe you have projected this problem to the point where one may speculate as much as one wishes. I am glad that you brought it up because it certainly deserves further discussion. What precisely is happening in these preparations from the enzymatic point of view? In the normal animal, the liver possesses a system of enzymes which is engaged either in synthesizing fatty acids from suitable material, or oxidizing them completely to carbon dioxide. When insulin is removed by pancreatectomy, fatty acid synthesis ceases, complete oxidation to carbon dioxide also apparently ceases, and an abnormal type of fatty acid oxidation ensues, resulting in an excessive production of ketone bodies. If the second hormonal principle is removed by hypophysectomy, as in the Houssay preparation, the liver apparently turns to an essentially normal type of metabolism with respect to the synthesis of fatty acids, and the absence of ketone formation. One is free to speculate as much as one pleases as to how insulin and the pituitary factors fit into the enzymatic picture. I have no answer and I would be delighted if you can furnish one or perhaps project experiments which would enable one to find the answer. I like to think of the relation of an enzymatic system to hormones as one which requires some sort of chemical combination between the hormones and the enzymatic system for normal activity. But in the livers of the Houssay preparation with respect to fatty acid synthesis and ketone formation, we have an example which shows that if you take away both of these hormonal factors which are required for normal activity the enzyme system appears to function in a very normal fashion. It raises a baffling problem as to the normal role of these hormones in terms of regulation of enzymatic activity.

DR. LEVINE: I would agree that the problem is baffling. In connection with this some recent experiments of Gurin indicate a definite *in vitro* action on lipogenesis in an isolated system.

leached from intact diaphragm in the course of exposure to medium in the Warburg vessel. I was wondering to what extent the lactate production observed may have occurred extracellularly as a consequence of such leaching, where glycogen formation could perhaps not occur in the free medium. Could the extraction from muscle of the enzymes during shaking have accounted, at least in part, for some of these results?

DR. STADIE: Naturally, Dr. Stetten, we were aware of the possibility you have mentioned as a source of error. We examined repeatedly in experiments of different types the possibility that glycolytic enzymes were leached from the diaphragm. We were never able to demonstrate any glycolytic activity in the medium following a period of equilibration with the diaphragms. I believe we have excluded this possibility as an explanation of the phenomena under consideration.

DR. LEVINE: Insulin does influence the amount of carbon dioxide derived from glucose. One would presume on the basis of the Meyerhof scheme, as we accept it now, that at least pyruvate would be an intermediate in this situation. Is there a contradiction between this and your data?

DR. STADIE: We have examined this possibility with some diligence, Dr. Levine, and have tried to demonstrate an effect of insulin upon CO_2 production by the rat diaphragm from isotopic glucose. Under the conditions of our experiments the insulin effect on carbon dioxide production is negligible.

DR. LEVINE: From your data dealing with fat metabolism, especially with the Houssay cat, and those of Brady, Gurin and Lukens (13), would you then conclude that the defect found in lipogenesis is a defect of the diabetic state, but is not due directly to insulin lack? The diabetic state is the result of removal of insulin from a balanced system, as you have indicated When both insulin and pituitary factors are removed, lipogenesis seems to proceed

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the yeast cells are changed by enzymatic action. Rothstein attributed this to enzymes upon the cell surface. Haugaard, in our laboratory, using rat diaphragms, showed that ATP, which is presumably unable to enter the diaphragm, is split by an ATP-ase action apparently localized to the surface of the diaphragm. Controls showed that this action was not due to leakage from the diaphragm of ATP-ase. 3) If this phenomenon occurs *in vivo*, it may have considerable physiological importance. It may permit one metabolic pathway to proceed without hormonal intervention while another pathway with similar enzymatic makeup would be hormonal responsive. 4) We have attempted to reverse the situation and restore insulin responsiveness to the lactic acid system. In preliminary experiments, we have been able to do this but prefer to study the problem further before discussion. In summary, this is a new phenomenon, but we believe our experiments show it to be a real one. It may be an artifact introduced by special conditions or it may be of real physiological significance. Only further experimentation can tell.

DR. WICK: In the eviscerated animal, in the complete absence of insulin, there is a substantial transfer of glucose, which is around 250 milligrams per kilo per hour. This goes on, even though there is apparently no insulin present. When you give insulin this is immediately increased, up to 500 milligrams per kilogram per hour. From what you said, it might be that the transfer in the absence of insulin is an entirely different mechanism from the extra 250 milligrams that occurs with insulin.

DR. RANDLE: There is one other thing I wanted to ask you, Dr. Stadie. Although insulin didn't increase the production of lactic acid, I presume that in your experiments you don't get complete conversion to glycogen of all the extra glucose taken up. This would suggest that there are some other compounds formed from glucose, under the influence of insulin, which are not glycogen or lactate.

DR. STADIE: I think that is true, Dr. Randle. We also cannot account for 100% of the isotopic glucose. Part of the missing glucose

DR. STADIE: I'm sorry but I have to contradict that. I have discussed this situation with Dr. Gurin. He states that he or his group have never demonstrated any action of insulin on fatty acid synthesis by these extracts.

DR. LEVINE: I don't know whether it was these extracts, but I believe there is one publication in which insulin was added *in vitro* to extracts which synthesized fatty acids and increased the synthesis. I may be absolutely wrong.

DR. STADIE: I have checked this very thoroughly with Dr. Gurin and I think the situation is as I have already stated it.

DR. RANDLE: I was interested in Dr. Stadie's remarks about the preferential action of insulin upon the synthesis of glycogen by the isolated rat diaphragm. I think that the work of Chain is pertinent to this observation since Chain obtained results which suggested that insulin might exert an effect on some extraphosphorylative pathway for oligosaccharide formation (Chain, A. B., Catamzaro, R., Chain, E. B., Masi, I., Pocchiari, F. and Rossi, C: *Proc. Roy. Soc. B., 143*:481, 1955). It is interesting to speculate on this. Now if the transfer of glucose across the cell barrier involves the formation of some intermediate, which in muscle is presumably not a phosphate derivative since glucose-6-phosphatase is lacking, it is possible that this glucose derivative might be polymerized to yield glycogen or oligosaccharide. It presumably, wouldn't go through the Embden-Meyerhof pathway because it would not be a glucose phosphate. This could provide one possible explanation of Dr. Stadie's remarks.

DR. STADIE: I wish there would be more discussion as to the significance of this dichotomy. May I run briefly through some possibilities: 1) The phenomenon may have no physiological significance. In other words, it may be an artifact induced by the special conditions of the *in vitro* systems in which the experiments were done. 2) However, the observation may not be entirely new. Rothstein has reported experiments using yeast in which metabolites in the medium which are assumed not to be able to penetrate into

glucose-6-phosphate in medium plus isotopic glucose, we cannot demonstrate a pathway by which the carbons of the glucose-6-phosphate in the medium appear in the glycogen. In contrast, they invariably appear in the lactic acid and the specific activities with *either arrangement become essentially equal.*

DR. WICK: We have administered phosphorylated compounds and found that they circulate only in the extra-cellular compartment, when using the eviscerated animal.

DR. STADIE: That is exactly what we found. For clarity, let me make a distinction between internal and external phosphate esters. The internal esters are isolated from the diaphragm proper; the external ones appear in the medium. With isotopic glucose in the medium, we were never able to demonstrate labeled fructose-1-6-diphosphate in the diaphragm proper, i.e., internal phosphate. In contrast, non-labeled fructose-1-6-diphosphate in the medium became labeled when the diaphragm was equilibrated with isotopic glucose. Its specific activity, when reisolated from the medium, was about the same as that of the lactate also isolated from the medium.

DR. WICK: If I understand you correctly, both lactate production and glycogen production are pictured as occurring intracellularly in the diaphragm, in other words extra-cellular production of lactate has been ruled out. This then leads to the conclusion, does it not, that there are two reservoirs in the cell, of hexosphosphate, which are not mixed at all, which are completely discrete, the one arising from extra-cellular glucose, and the other arising from —what? We have two reservoirs then of intracellular hexosphosphate within the muscle cell which are not mixing, the one leading to glycogen and the other leading to lactate.

DR. STADIE: As I picture it, there is one pathway by which glucose goes into the cells of the diaphragm where it is phosphorylated, yielding in turn glucose-6-phosphate, glucose-1-phosphate, or glycogen. These internal phosphate esters cannot be washed out of the diaphragm. This pathway is influenced by insulin.

may be incorporated by transamination into protein. It is possible, by chromatographic methods, to demonstrate radioactive alanine originating from isotopic glucose in these diaphragms. I think that is what the results of Sinex, McMullen and Hastings suggest. They studied the incorporation of radioactive alanine into the proteins of the isolated rat diaphragm under the influence of insulin. The addition of unlabelled pyruvate lowered the specific activity of the diaphragm proteins, and thus appeared to inhibit the incorporation of labelled alanine. The authors did point out the alternative explanation, namely that the specific activity of the alanine may have been lowered by dilution with unlabelled alanine derived from transamination with unlabelled pyruvate (Sinex, F. M., McMullen, J. and Hastings, A. B.: *J. Biol. Chem.*, *198*:619, 1952).

DR. LEVINE: Villee and Hastings at one time accounted for the C^{14} glucose disappearing under the influence of insulin to the extent of about 80%, in terms of glycogen, pyruvate, carbon dioxide, lactate, etc. (14). As I remember it 80 or 85% of the sugar was accounted for.

DR. STADIE: I think that possibly that is true. I remember the paper.

DR. WICK: I had been under the impression that it could not get into the cell.

DR. STADIE: I do not believe that the glucose-6-phosphate gets into the diaphragm. When isotopic glucose-6-phosphate is in the *medium* and equilibrated with the diaphragm, and then reisolated from the *medium* together with lactate, they both are found to have the same specific activity. In contrast, none of the radioactivity of the glucose-6-phosphate showed up in the glycogen isolated from the diaphragm proper. That's the point I am trying to make. In other words, by any arrangement, viz, isotopic glucose-6-phosphate in medium plus nonisotopic glucose or nonisotopic

[14] VILLEE, C. A AND HASTINGS, A B. *J. Biol Chem.*, 179 673, 1949.

DR. LEVINE: I think that we ought to clear up confusion about terminology. In order to be neutral as to what the mechanism is, one should use a term which does not have any other connotations. Unfortunately, we didn't abide by that in our first publication, and we used "permeability." Since the term permeability has definite connotations, this has justifiably become confusing in the minds of many people. We were careful thereafter to call it a transfer system, or as Dr. Stetten calls it, translocation. This term does not imply that it is either permeability, or diffusion, or any other physical process. Because of the degree of specificity shown, this intricate system has all the ear marks of an apoenzyme; that is a protein with a definite structure into which certain sugars would fit. What happens in the course of transfer we do not know. All we can say is that on the other side we can recover free galactose. That does not mean that there has not occurred a subtle change in between. In other words, I think that we would both agree, Dr. Wick, that this is not diffusion, and I want to be on record as saying that this phenomenon does not exhibit the characteristics of diffusion Whether this is an enzyme, in the usual sense in which we use the term enzyme, to mean a transformation of the molecule into something else, a derivative, I don't know. Sometimes in fantasy I picture it as half an enzyme. Suppose there were at the surface of the cell or near it a protein or lipoprotein which would combine with certain sugars (hexoses and pentoses) of definite structure. Then instead of this leading to a change in substrate, it leads to a physical change in the protein-substrate combination. This complex may either contract or twist and then the molecule of sugar finds itself 50 angstroms away from its original position. This is of course a naive picture, but I do want to set the record straight as not implying that the transfer of sugar means simple diffusion.

DR. WICK: I would like to go one step further and compare substrate specificity of brain hexokinase and the specificity requirements for insulin responsiveness which we have observed in the eviscerated-nephrectomized rabbit. Representative compounds that do not respond to insulin are sorbitol, gluconic acid, glucuronic acid, fructose and 3-methylglucose. On the other hand, galactose, mannose, glucosamine and 2-deoxyglucose respond to insulin

DR. STETTEN: But they do get into the cell, and extracellular hexosphosphate is a precursor of lactate, or isn't that necessary.

DR. STADIE: I think it is necessary.

DR. STETTEN: Is this hexosphosphate distinct from the hexosphosphate that the cell makes; that is by the nexokinase reaction?

DR. STADIE: Dr. Stetten, I can only answer your question by speculation. I picture the enzymatic system concerned with the formation of lactate from medium glucose as not having a barrier to glucose which is raised by insulin. Apparently also the system has no barrier to the ready entrance of phosphate esters such as glucose-6-phosphate. Accordingly, we find that glucose-6-phosphate in the medium mixes with the glucose-6-phosphate formed from the isotopic medium glucose, and acquires the isotopic label. Presumably this means that it mixes with the glucose-6-phosphate pool formed by enzymatic action. In contrast, the pathway of glucose to glycogen in the diaphragm has a barrier which is lifted by insulin, so that extra glycogen is formed. In addition, this enzymatic system presents a barrier to phosphate esters. Accordingly, these esters neither penetrate into the diaphragm, nor do they escape from the diaphragm into the medium.

DR. STADIE: I think we showed that all the Embden-Meyerhof reactions occurred. Let us use the word external and internal metabolite in the sense that Lehninger uses these terms in his discussion of mitochondrial reactions. External glucose-6-phosphate and external fructose-1-6-diphosphate become labeled if the equilibration of the diaphragm is done in the presence of isotopic glucose. The specific activity becomes essentially the same as that of the lactate. Now whether these phosphates went into the cell and came out again on the surface, I have no way of telling. In contrast, we never found that the internal fructose-1-6-diphosphate was labeled.

DR. WICK: Dr. Stadie, I think it is in order to bring in this idea of permeability or diffusion. You suggest that glucose goes into the cell as the free form, and thus agree with Dr. Levine's theory.

DR. LARDY: I would like to say something about the hexokinase specificity. The enzyme that Crane and Sols have studied, is the enzyme of brain, a tissue that does not respond to insulin. No one has ever isolated the hexokinase of peripheral tissues. Extracts have been obtained which show the activity, but the exact nature of that reaction is not certain. One hexokinase which has been studied is the fructose-kinase of liver. This again is a metabolite which presumably does not respond readily to insulin, so that you can't draw too many conclusions about the specificity of substrate structure in the insulin response, and its relation to the hexokinase, until we know what hexokinase really is like.

I have a question to ask of Dr. Stadie. It appeared that the counts in lactic acid were very low when you used uniformly labeled glucose. Can you tell us how many micromoles of lactic acid are produced by one diaphragm?

DR. STADIE: From 100 to 200 micromoles per gram for two hours.

DR. LARDY: And how does that compare with the amount of glycogen synthesized?

DR. STADIE· Very much greater. Special conditions are used for equilibration. The pH is 7.4, the phosphate is higher than is customary, namely, 0.05 molar. As Zapp and I showed years ago, lactic acid formation by a diaphragm from glucose is a function of pH. If one lowers the pH of the medium to 6.8, lactic acid production becomes zero, particularly if phosphate is cut down to about 0.02 molar. We deliberately chose the above condition, i.e., pH 7.4, phosphate 0.05 in order to accentuate lactic acid formation from medium glucose. However, the phenomena which we are discussing may be demonstrated within a pH range from 7 to 8.6.

DR. ASTWOOD Regarding the portion of Dr. Stadie's paper dealing with oxidative phosphorylation, are there any findings which would be inconsistent with insulin action being confined to the transfer system?

DR. STADIE It seems to me that what you're faced with is the problem of explaining two actions of insulin. One upon the early

administration in our preparations. It is apparent that insulin action is fairly specific. Sols and Crane (*J. Biol. Chem., 210:*58, 1954) have concluded that specificity of brain hexokinase involves the ring structure and the hydroxyl groups at the carbon atoms 1, 3, 4, and 6 of the glucose molecule. With the exception of galactose, it is apparent that the specificity requirements for insulin action is similar to those reported by Sols and Crane.

DR. LEVINE: Dr. Wick, in addition to galactose, l-arabinose and d-xylose may be transferred and these sugars also are not phosphorylated. Dr. Weil-Malherbe in his recent review (15) on insulin action, reviews the specificity which we had indicated, i.e., your work, that of Wilbrand in the red cell, and that of Sols and Crane for hexokinase. His conclusion is that the specificity of the in sulin response for sugars is totally different than that of Sols and Crane for brain hexokinase. Therefore, he would lean towards a reaction preceding hexokinase.

DR. RANDLE: If insulin acts on a phosphorylation of some sort, then insulin ought to stimulate the uptake of glucose by muscle under anaerobic conditions if glycolysis can provide sufficient ATP. Evidence has been presented suggesting that insulin does increase the uptake of glucose by muscle under anaerobic conditions (Walaas and Walaas: *J. Biol. Chem., 195:*367, 1952, Ottaway, J. H.: *Biochem. J., 61:*441, 1955). However, an effect of insulin in the absence of oxygen could only be demonstrated with short periods of incubation, and there may have been sufficient oxygen in muscle for the conditions to have been not truly anaerobic.

Insulin has an action on the incorporation of amino acids into protein in the absence of added oxidizable substrate such as glucose (Krahl, M. E.: *J. Biol. Chem., 200:*99, 1953; Sinex, F. M., McMullen, J. and Hastings, A. B.: *J. Biol. Chem., 198:*619, 1952). This suggests either that insulin has an intracellular action on the metabolism of carbohydrate, or that insulin has a direct action on the transfer of amino acids across the cell wall or the incorporation of amino acid into tissue protein.

[15] WEIL-MALHERBE, H. *Ergebn. d Physiologie*, 1955

DR. LARDY: I would like to say something about the hexokinase specificity. The enzyme that Crane and Sols have studied, is the enzyme of brain, a tissue that does not respond to insulin. No one has ever isolated the hexokinase of peripheral tissues. Extracts have been obtained which show the activity, but the exact nature of that reaction is not certain. One hexokinase which has been studied is the fructose-kinase of liver. This again is a metabolite which presumably does not respond readily to insulin, so that you can't draw too many conclusions about the specificity of substrate structure in the insulin response, and its relation to the hexokinase, until we know what hexokinase really is like.

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DR. ASTWOOD: Regarding the portion of Dr. Stadie's paper dealing with oxidative phosphorylation, are there any findings which would be inconsistent with insulin action being confined to the transfer system?

DR. STADIE: It seems to me that what you're faced with is the problem of explaining two actions of insulin. One upon the early

stage of carbohydrate metabolism (the translocation theory is now in ascendant); and the other is the effect of insulin upon fatty acid metabolism. As far as I know no one has proposed a theory which indicates that oxidative phosphorylation and the supply of ATP may conceivably explain either or both. I see nothing contradictory in the two concepts.

DR. STETTEN: I think it is generally agreed that insulin favors the accumulation of hexose phosphate esters. It makes these available for other purposes. To make glucose-6-phosphate at least three components are required. One of them is intracellular glucose. This is the component whose concentration would be favored by Dr. Levine's picture. The second is hexokinase. This is the component which would presumably be favored as the crucial component in the Cori picture. The third is ATP and this may be the component which Dr. Stadie favors as the crucial component. The difficulty with ATP, is that in addition to being a necessary agent for the formation of glucose-6-phosphate, it is also an abundant product of this compound in the biological breakdown. Therefore, it must ultimately be ascertained whether the defects in phosphorylation which have been shown by [illegible] s, and have been indicated [illegible] r people [illegible] and its [illegible] reflections of the primary def[illegible] to me unlikely that the defect in phosphorylation is a primary defect, for two reasons. First, as was mentioned here, Dr. Lardy has shown that the defect in oxidative phosphorylation in the alloxanized rat liver mitochondria was not manifest. Whereas it may be true that these anim[illegible] tic, they are certainly [illegible] manifest is manifested [illegible] apparently normal oxidative phosphorylation. Second, we know more or less what an animal with uncoupled oxidative phosphorylation looks like. That is the dinitrophenol-poisoned animal which clinically is much more similar to the thyrotoxic than to the diabetic animal.

DR. LARDY: No one has mentioned the defect in fat synthesis. Many of the people who have worked in this field have completely

overlooked the papers that Dr. Foley in England published many years ago. He pointed out that in order to have fat synthesis at any appreciable rate, it was necessary to have some glycerol around. I believe you can explain some of these effects by assuming that the diabetic animal has less hexose phosphate esters around which can be precursors of alpha glycerol phosphate, and would be a means of leading the fatty acid away from co-enzyme A. Now in the experiments that you cited, those of Drs. Shaw and Gurin, where butyryl co-enzyme A was added, it is possible that with an abundance of co-enzyme A, condensations of butyryl groups can occur and the longer fatty acids formed may still remain on the co-enzyme A molecule. I am wondering if they did experiments in which an abundance of acetyl co-enzyme A was added to see whether that would give rise to fatty acid synthesis.

DR. STADIE: I believe that acetyl-CoA was without effect in preparations from diabetic animals.

DR. LARDY: In the same amount?

DR. STADIE: *I can't answer that. However, I should like to suggest* to Dr. Lardy that the glycerol hypothesis must meet the objection that fructose in the diabetic animal goes through the Krebs cycle, producing carbon dioxide in unaltered amount compared to the normal. Presumably, it is a source of glycerol, but notwithstanding this, it does not ameliorate this defect of fatty acid synthesis.

DR. LARDY: I was of the opinion that Dr. Chaikoff found that it did.

DR. STADIE: Only if you feed it to the intact animal. That's quite a different proposition. When the liver slice from the diabetic is equilibrated with fructose, the fructose is oxidized to carbon dioxide but it does not restore the ability of the slice to incorporate acetic acid, lactic acid, or pyruvic acid into higher fatty acids. In addition, the fructose itself is not synthesized to fatty acids.

DR. LARDY: In the diabetic slice you may have depleted the system of enough ATP to prime the fructose phosphorylation. There

may be a generalized metabolic deficiency in the diabetic slice as a result of depletion of available carbohydrate.

DR. STADIE: It must be pointed out that these fatty acids isolated by Gurin and his coworkers are not in the triglyceride form.

DR. LARDY: No hydrolysis is carried out?

DR. STADIE: None whatever.

DR. RANDLE: Folley has demonstrated an *in vitro* effect of insulin on lipogenesis in mammary gland slices (Balmain, J. H., Folley, S. J. and Glascoch, R. F.: *Biochem. J.*, *56*:234, 1954).

DR. WICK: Dr. Stadie, you say that lactic acid did not come from glucose, is that correct?

DR. STADIE: No. Eighty to 90% of it comes from glucose. About 75% of the extra glycogen synthesized in the presence of insulin also comes from the medium glucose. I would like to quote a favorite philosopher of mine. I think it was Occam who said: "To all situations apply the razor; and leave but one hypothesis." If you must select one single hypothesis and bet upon it for what the future will bring in regard to the problem of insulin action, oxidative phosphorylation would be a good one to put your money on. At least it is conceivable that all the diabetic defects which have been demonstrated might involve oxidative phosphorylation. However, the evidence to integrate these phenomenon into one convincing picture is missing.

DR. WICK: I have some material on 2-deoxyglucose, a compound that may prove to be very useful in insulin studies. When we recently reported on the insulin responsiveness of 2-deoxyglucose in the eviscerated animal (*Proc. Soc. Exper. Biol. & Med. 89*:579, 1955), we concluded from its overall metabolic effects that it blocked the glucose transfer mechanism. A metabolic block of this type has never before been described. The following table shows

some overall metabolic effects resulting from a single injection (100 mg./kilo) of this glucose analog.

Single Injection Effect of 2-Deoxyglucose (100 mg/kilo)

Exp. No.	1	2	3	4
Glucose Used				
Before Deoxy	450	440	460	540
After Deoxy	210	260	172	300
Expired CO_2				
Before Deoxy	1005	1067	780	830
After Deoxy	413	634	420	530
Glucose Oxid.				
Before Deoxy	213	220	189	—
After Deoxy	117	138	92	—

All values expressed as mg/kilo/hr Three hour averages.

In these experiments the animals were followed for a three-hour period before 2-deoxyglucose administration and then followed for another three-hour period after its administration. Maximal insulin dosage was given throughout, and the blood sugar was maintained at 100 mg/100 ml. By this procedure each animal acts as its own control. All of the values are expressed as mg/kilo/hr., and are the average values for the corresponding three-hour periods. It is apparent that 2-deoxyglucose reduces the sugar requirements of these animals. The reduction occurs within 30 minutes, which is the time required for the 2-deoxyglucose to enter the cells of the extrahepatic tissues. Thus, this glucose analog successfully competes with glucose for cell entry. The drop in glucose requirement is not a temporary one, but persists as if a permanent block has taken place. It is obvious that the sugar reduction is not due to a simple substitution of fuels or the conversion of 2-deoxyglucose to glucose. The data also show that there is a marked reduction in the output of carbon dioxide and in glucose oxidation. It is apparent from these results that 2-deoxyglucose is a metabolic block for glucose metabolism.

In order to gain more information regarding the nature of the block, we examined the effect of 2-deoxyglucose on the oxidation of acetate and these results are shown in the following table.

Single Injection Effect of 2-Deoxyglucose (200 mg/kilo)
On Acetate Oxidation

	Three Hour Averages	
	Before Deoxy	After Deoxy
Expired CO_2 mg/kilo/hr.		
Glucose used	480	243
Acetate oxidation cts/hr	181,000	192,000
Acetate given by constant injection (100 mg/kilo/hr.).		

In the above experiments the analog was given as a single injection, and the labeled acetate was given by constant infusion at the rate of 100 mg/kilo/hr. The animal was followed for a three-hour period before and after the administration of the analog. The data show that 2-deoxyglucose had the typical effect on the expired carbon dioxide and glucose disappearance, but it had no effect on the oxidation of acetate. The results indicate then that 2-deoxyglucose acts as a block—not in the oxidative area—but rather in the glycolytic area.

We also know by employing C^{14} labeled 2-deoxyglucose that the compound is not oxidized to carbon dioxide in any significant amounts.

With respect to the localization of the block in the glycolytic area, we have some good leads resulting from the work of Drs. Nakada and Wolfe of our laboratory. These workers have confirmed the work of Sols and Crane, showing that 2-deoxyglucose is phosphorylated by brain hexokinase to form 2-deoxyglucose-6-phosphate. Nakada and Wolfe have also shown that 2-deoxyglucose-6-phosphate is inhibitory to phosphoglucoisomerase. The most likely site for the block is, therefore, at the isomerase stage in our experiments. In this reaction the ring shifts from the pyranose form between carbon atom 1 and 5, to the furanose form between carbon atoms 2 and 5. Since 2-deoxyglucose does not have a hydroxyl group on the #2 carbon, it is most likely that the corresponding step involving 2-deoxyglucose-6-phosphate to the fructose compound cannot take place. One can picture this block at the isomerase stage as the primary block, which then results in a chain of events leading to a reduction in the transfer rate of glucose. A secondary block could be the accumulation of glucose-6-phosphate which is itself inhibitory to the hexokinase reaction. If

insulin action is concerned mainly with the intracellular transfer of glucose and independent of the hexokinase reaction, then one would expect free glucose to accumulate in the cells. In our preliminary experiments the glucose we have found can be accounted for by that circulating in the extracellular compartment.

We have also examined the effect of 2-deoxyglucose on galactose transfer and found that the galactose transfer was uninhibited. These results could be interpreted to mean that the mechanism for galactose transfer is unlike the transfer involved for glucose. However, additional work will have to be carried out to prove this.

DR. LARDY: I have one question that I would like to direct to Dr. Wick. If insulin enhances the formation of glycogen in the diaphragm from either glucose or fructose, why doesn't it have any effect on fructose penetration into the tissues of the eviscerated animal?

DR. LEVINE: When fructose is injected into the eviscerated animal, one does not get the same type of curve that one gets with a non-utilizable sugar. A non-utilizable sugar will distribute in a particular compartment and its concentration will level off. Fructose is utilized to a certain extent by the eviscerated preparation. The rate is about a tenth of that which occurs in the presence of the liver. The liver takes up fructose very avidly; the other tissues very much less so. The prior injection of insulin and the continuous injection of insulin (in the presence of 100 milligrams per cent glucose level) does not exert any influence on the curve of fructose distribution and utilization. There is no difference between the insulinized and the non-insulinized animal. We have also done fructose infusions in the virtual absence of blood glucose, by waiting until the eviscerate preparation had a glucose level of 5 to 10 milligrams per cent Then the fructose was given. Under these circumstances insulin still had no significant effect on the distribution values of fructose (16). I do know that in the rat

[16] GOLDSTEIN, M. S, HENRY, W. L, HUDDLESTUN, B AND LEVINE, R: *Am J. Physiol*, 173 207, 1953.

diaphragm Rothstein (17), Mackler and Guest (18), as well as Mirsky, *et al.* (19) showed insulin effects on fructose uptake in the absence of glucose. This is not the case in the dog. We do not understand the difference between our laboratory and that of Drury and Wick in relation to insulin action on mannose utilization. In the rat and in the dog mannose levels are not effected by insulin. In the eviscerated rabbit (Wick and Drury), they are. The nonresponsiveness of mannose to insulin was also shown many years ago by Cori in the rat (20). Therefore, one must invoke species differences in the sugar transfer system.

DR. STETTEN: I would like to ask a question of the clinicians. In this agent 2-deoxyglucose we have a material which markedly impedes the utilization of glucose by muscle, and which in its toxicology does not produce convulsions, and therefore presumably does not interfere with the nutrition of the central nervous system. Is it perhaps therapeutically useful in hyperinsulinism?

DR. WICK: The renal threshold for 2-deoxyglucose in the rat is very low.

DR. STADIE: Dr. Wick, I didn't quite get something, and I wish you would straighten me out. Is it your hypothesis that deoxyglucose affects translocation or transfer?

DR. WICK: The overall effect is a decreased transfer of glucose.

DR. STADIE: Is it localized on what we call the transport system?

DR. WICK: It didn't affect galactose transfer so that its effect on the transfer mechanism may be an indirect one.

DR. STADIE: I am puzzled by this. According to Hastings the intraglycolytic system, that is once past the initial block up and down,

[17] DENNIS, D. J AND ROTHSTEIN, A: *Am. J Physiol*, *178*.82, 1954

[18] MACKLER, B. AND GUEST, G M. *Am J. Physiol*, *174* 487, 1954

[19] HAFT, D, MIRSKY, I A. AND PERISUTTI, G: *Proc Soc Exper. Biol & Med*, *82*·60, 1953.

[20] CORI, C F AND CORI, G. T: *Proc Soc Exper. Biol & Med*, *26*.432, 1929

pyruvate to glycogen or from glycogen down to pyruvate, seems to be unimpeded in the diabetic. That's my interpretation of his data, and I think that is about his conclusion. If so, then considering the action of deoxyglucose upon an isomerase, what would its relation to insulin action be, since insulin apparently has no concern with that reaction. In other words, is deoxyglucose a cytotoxic agent, which happened to localize its action on one particular enzyme?

DR. LARDY: I think it would appear that the 2-deoxyglucose may inhibit the phosphorylation of glucose, and the conversion of glucose to glucose-6-phosphate. That step would be in the area which Hastings assumes is not influenced by insulin. I don't think that there is a dichotomy between these two concepts.

DR. LEVINE: In other words, Dr. Lardy, you postulate two points at which deoxyglucose inhibits, one the isomerase, and the other the kinase?

DR. LARDY: Not necessarily. The isomerase may be the point at which the inhibition of the hexosekinase is exerted. If you have accumulation of glucose-6-phosphate, because the isomerase can't remove it, you have a product which inhibits at least brain hexosekinase, perhaps it inhibits muscle hexosekinase too.

DR. RANDLE: I think Dr. Wick that you said that in the presence of 2-deoxyglucose, glycogen content was markedly increased. Could this be because the breakdown of glucose-6-phosphate to pyruvate is blocked, and so the glucose-6-phosphate is shunted to glycogen?

DR. LARDY: The total amounts of glucose disappearing are tremendously reduced, even though you are getting more glycogen formed. The additional glycogen formed certainly does not account for the amount of glucose metabolized to carbon dioxide under ordinary circumstances. Isn't that right, Dr. Wick? That is as shown by your much decreased glucose disappearance.

DR. WICK: At no time is there complete conversion of glucose to expired carbon dioxide. Ordinarily only about 25 to 50% of the glucose that disappears from the plasma is recovered as labeled expired carbon dioxide.

DR. LARDY: The point I was trying to make is that in the presence of 2-deoxyglucose you do not shunt the entire glucose utilization to glycogen. The amount of glycogen formed in the presence of 2-deoxyglucose must be small compared to the amount of glucose that was previously used, even though it may be great in comparison to the amount of glycogen which had been there previously. How much glycogen is in the muscle of the eviscerated animal treated with 2-deoxyglucose?

DR. WICK: The presence of 2-deoxyglucose does not shunt all of the disappearing glucose to glycogen, although it is our impression from three or four experiments, that a high muscle glycogen content is found after administration of 2-deoxyglucose. We do not have sufficient number of experiments to give actual values.

DR. LEVINE: Dr. Wick, your data on 2-deoxyglucose show an inhibition of the total utilization of glucose, or disappearance, in the ordinary terminology. The block is presumably at the isomerase stage. Therefore, glucose might enter, become glucose-6-phosphate, glucose-1-phosphate, glycogen, or perhaps take other pathways. Under these circumstances, it is not surprising that, even with insulin, there would be no free intracellular glucose found, because the transfer system would still be affected by insulin and transformation to glucose-6-phosphate and glycogen could occur. Free glucose would only be found if the inhibition were at the hexokinase step, as Park has shown. Your data show that deoxyglucose did not interfere with the transfer of galactose, which is consistent with this interpretation.

DR. BEST: At the conference we had in New York, didn't Dr. Cori say that there were no methods available to distinguish between the transfer phenomenon and the primary action on hexosekinase?

DR. STADIE: I think that Park was the first one to devise experiments which could distinguish the action of insulin upon the transport mechanism and the hexokinase reaction in the same system. Therein lies the beauty of this concept. At 38°, he saturated the hexokinase system by elevating the medium glucose; or he inhibited it in whole or in part by lowering the temperature to 15°. To my mind, that was a brilliant contribution. Before the introduction of Park's concept, the only way to differentiate between th action of insulin on transport or on hexokinase, was to demonstrate the action of insulin upon cell-free extracts. Such a demonstration would conceivably eliminate the transport hypothesis. Unfortunately a Scotch verdict, i.e., finding no action of insulin with extracts, meant very little because it can always, be said that some fragile inhibitor had been lost during the preparation.

DR. WICK: I would like to ask Dr. Stadie if he doesn't think that it is possible to have cell leakage when the glucose concentration of the medium is 2000 mg. per 100 cc.? Lowering the temperature may also alter the normal permeability characteristics of the cell wall.

DR. STADIE: I think, Dr. Wick, that those are reasonable objections, but if I may be amicus curiae to Park, I believe that these objections could be met as follows: On one hand, you suppose that high temperature disintegrates the transport system, and on the other hand, you say that low temperatures produce the same effect. It seems unlikely that high temperatures plus high glucose concentrations would disable a system and that low temperatures with low glucose concentrations would do the same thing.

DR. RANDLE: I think that Chain's experiments suggested very strongly that insulin was not affecting phosphorylation and that oligosaccharide could be formed without prior phosphorylation of glucose. He studied the fate of labeled glucose in diaphragm under the influence of insulin in very short incubations. He could find labeled glucose in oligosaccharide before it appeared in glucose phosphates. If the action of insulin was on the phosphorylation of glucose, then glucose phosphate should have been labeled before oligosaccharide appeared.

DR. LEVINE: May I also point out that if 2000 mg. percent glucose had disrupted the membrane, then there shouldn't be any insulin effect, but Park showed a difference between no insulin and insulin.

DR. KINSELL: If there is a lull in this particular discussion, I wonder if some of the members of the conference would care to give some consideration to fat transport mechanisms. The evidence is most convincing that under normal conditions, a great portion of the total energy in the mammalian organism is derived from fat, with the acetate being the material that is combusted in greatest amount at the cellular level. This brings one to the question as to what the probable materials which travel from fat depot, to liver, to muscle, are. Among the possibilities would be fatty acid, ketones, and acetate per se. Since Dr. Wick is one of those who has been very active in this field, I wonder if he would like to comment.

DR. WICK: Before the use of isotopes it was generally thought that injected glucose was rapidly oxidized to carbon dioxide and water. Work with labeled glucose in many laboratories has shown this is not the case. In the eviscerated rabbit the rate of oxidation of the glucose, as determined from the amount of tagged carbon in the expired carbon dioxide, increases slowly; and even after six hours of maximal insulin activity, only half of the disappearing glucose is oxidized. At this time it can be calculated that approximately 40% of the expired carbon dioxide is coming from the injected glucose and its derivatives. Maintaining the animals for 24 hours will not increase this value more than 5 to 10%. Likewise, it can be calculated that 30 to 40% of the expired carbon dioxide is coming from non-glucose metabolites even when the blood sugar is maintained at 1000 mg. per 100 cc. over an eight-hour period. In the absence of insulin administration, only 15 to 20% of the expired carbon dioxide is coming from glucose or its derivatives. These results indicated that there are pools of different metabolites in the body which are constantly being oxidized despite a high concentration of intracellular glucose derivatives. These results are not characteristic of only the eviscerated animal. Comparable con-

ditions have been reported in rats, dogs and man. The source of the carbon atoms not arising from glucose is of great interest to us.

DR. STETTEN: We are frankly puzzled by the whole dilemma, as I suspect every one else is, and frustrated by our inability to demonstrate the precursors for the carbons in carbon dioxide, in the intact animal. We assumed it was so simple initially. We have done some tentative experiments injecting acetate and acetoacetate into such animals, but the total yields of carbon dioxide are vanishingly small and the results are hard to interpret.

DR. ASTWOOD: I have always wondered too why it is not easier to raise the RQ above one by infusing glucose. One would think that if all the glucose were turnd to fat one would get very high values. I have always been impressed with how difficult it is to get it even up to one, let alone high above one.

DR. STETTEN: I suppose that during the fattening process on a glucose intake you would get respiratory quotients in excess of one.

DR. LARDY: Dr. Stetten, did you and Dr. Ingle compare the conversion of glucose to the carbon dioxide in animals that were worked, as compared to those which were not worked?

DR. STETTEN: These were all anesthetized animals, but we have done experiments in which one or both hind legs were exercised in a fashion that Dr. Ingle has described. Under these circumstances, as was anticipated, the contribution of glucose carbon to carbon dioxide rose, if I recall, to values of about 60%, as against 20% in the resting, or rather anesthetized, non-worked animal

DR. LARDY: I think that those results strengthen current hypotheses regarding the role of inorganic phosphate and ATP in regulating the breakdown of carbohydrates. In the resting animal inorganic phosphate in the cell is kept at a low concentration. The tendency would be to drive any carbohydrate to glycogen. Fat

therefore might be oxidized somewhat preferentially. Now, when the animal works, there is an accumulation of inorganic phosphate in the muscle, which permits carbohydrate to be broken down to pyruvate and lactate. Thus it contributes to the CO_2 pool more heavily.

DR. WICK: In the eviscerated animal the lactate production is very high. The plasma levels of lactate remain uniformly high—around 100 mg/100 ml. We have found by injecting C^{14} labeled lactate that the turnover rate is very fast, and it is our belief that a significant fraction of the carbon dioxide in our preparations, at least, comes from the oxidation of lactate.

DR. GRIFFITH: Are we to infer that metabolism during work, during actual contraction of muscle fibers, is not just an exaggeration of the basal metabolism, but is entirely different process and under different control?

DR. STETTEN: In these anesthetized, resting animals, which are definitely sub-basal by ordinary standards, probably a very small fraction of the carbon dioxide is coming from the muscle at all, and much of the carbon dioxide that we are measuring is cerebral, renal, or hepatic. In the working animal a much larger fraction is derived from muscle, where the metabolic pathway is somewhat different than it is in liver, in which, as far as we know, the glycolytic pathway predominates.

DR. GRIFFITH: Because brain and nerve tissue use glucose primarily, is it not true that respiratory quotient data suggest strongly that carbohydrate is not the main fuel in the muscle in the resting animal?

DR. STETTEN: Yes I don't know what the resting animal is burning, but it is burning something that it contained *at the initiation* of the experiment.

DR. BEST: Dr. Stetten, would you like to present some new material?

DR. STETTEN: These are studies by Dr. James Field and myself, which represent an extension of observations made originally in Dr. Stadie's laboratory by Marsh and Haugaard.

Sera from normal subjects were diluted either with buffer or with buffer containing insulin; hemidiaphragms of rats were immersed, then removed from these media, and washed by Dr. Stadie's technique, and finally incubated for 90 minutes in glucose-Ringer's solution. The total glycogen content was determined, and in general insulin treatment resulted in a difference of the order of 5 micromoles of glucose equivalent per gram of tissue.

Sera from patients admitted to various hospitals in diabetic acidosis were studied. In most of the cases the insulin effect noted above was abolished or greatly decreased. A short time after the acidosis was controlled, the "insulin inhibitor(s)" usually disappeared from the plasma. In one patient, a diabetic who was allowed to relapse into acidosis under observation, within 10 hours after the institution of treatment we were unable to detect insulin antagonist activity in his serum.

We secured a sample of plasma from a rather remarkable patient in Charlottesville, thanks to Dr. Mulholland of that city. A previously mild diabetic was admitted to the hospital in profound coma, apparently precipitated by a severe pelvic infection. She was at once recognized as an extremely insulin-refractory patient, and within a few hours insulin administration was at the level of 1000 units an hour. She received 21,000 units of insulin within a 24 hour period, at which time she died. A relatively large sample of post-mortem blood was made available to us. Serial dilutions of this serum were conducted. From the dilution figures, and from the quantity of insulin in our *in vitro* assay system, it was possible to approximate that in the total serum of this patient at the time of death, there was enough antagonist to inactivate 10,000 units of insulin. It is therefore not surprising that she failed to respond to one thousand units of insulin administered hourly. The material which we have studied from this patient was apparently moderately thermostable. Heated for 15 minutes at 60 degrees, it was at last not entirely inactivated.

We have seen two patients who, though admitted in diabetic acidosis, failed to show serum insulin-inhibitor, which we now

have seen in eight or nine sera. Both of these patients, though in fairly severe acidosis responded rather readily to insulin, requiring approximately 200 units of insulin within the first 24 hours. We were interested in these cases because they showed an exorbitant level of compound F in the serum. We are inclined to believe that our activity is not of adrenocortical origin for this reason, and also for the reason that adding compound F to normal serum does not abolish the insulin effect. By injecting ACTH in rather massive amounts into a diabetic patient rise in blood compound F level occurred, but this serum did not abolish the insulin effect.

Two studies were conducted on patients in uremic acidosis. These patients were in severe acidosis but their sera were normal with respect to lack of insulin antagonist.

An experiment was performed in which radioactive iodo-insulin was tested, as Dr. Stadie has tested this material with respect to the capacity of hemidiaphragm to bind radioactivity. We could demonstrate no interference with the absorption or binding of iodo-insulin by diaphragm brought about by inhibitory serum. We have collected a small amount of additional information about this material. In addition to being relatively thermostable, it is non-dialyzable. Our inhibitory serum shows no insulinase activity, as measured by the method that Mirsky and others have employed. The material migrates electrophoretically in such a fashion that the serum albumin is completely devoid of activity. The activity is smeared rather badly throughout the globulin fraction, but the greatest abundance of activity appears to be in the *alpha-globulin* section of the paper curtain electrophoresis preparation which we have now secured. On the basis of the rapid disappearance of this activity after treatment is instituted, we are inclined to believe that this is not an antibody. We are inclined, as indicated, to believe that this is not insulinase; that it is not related, directly at least, to the activity of the adrenal cortices. There remains the possibility that this material may be of anterior pituitary origin. This is about as far as the work has gone to date.

DR. BEST: But its characteristics are not the same as those of Bornstein and Krahl's material?

DR. STETTEN: We originally felt that it was different from the material of Bornstein, in that it could be preserved in the frozen state. We have since had occasion to re-thaw and re-freeze a number of times samples which we knew were active in this regard. The activity may diminish on repeated refreezing and re-thawing. Centrifugally the material does not behave as a lipoprotein.

DR. GRIFFITH: May I ask if it is possible to demonstrate this material in the serum of a normal subject 30 to 40 minutes after the administration of a small amount of insulin, enough to stimulate the anterior pituitary and to lower the blood sugar to 50 or 60 milligrams percent?

DR. STETTEN: We have not tried that. We have been unable to demonstrate the material in thermally stressed animals, a condition which in some laboratories at least produces a degree of insulin resistance.

DR. STADIE: This is a subject which interests me very much; I should like to hear the clinicians' comments. I think the term insulin resistance is used in two senses, on the basis, perhaps, of the mechanism of generation. In one sense, the term is associated with patients, like those of Dr Stetten, who suddenly develop a severe ketosis, usually due to infection. They require amounts of insulin much larger than those which have hitherto controlled them. As a rule, I believe, these amounts are not excessive, perhaps ranging as high as 1000 or 2000 units per day. The basis of this condition is not understood, but as Dr. Stetten has shown, an evanescent inhibitor, if you want to call it that, appears in the serum, and then disappears upon restoration of glucose tolerance to normality. But there is a second type of insulin resistance, presumably based upon immunological disturbances. Marsh and Haugaard* from our laboratory had the opportunity of studying an interesting case in association with Dr. Asper at Johns Hopkins Hospital. This male diabetic who was controlled without insulin, and who had no ketosis or no infection, suddenly developed a

* Marsh, J D, and Haugaard, N: *J. Clin Investigation, 31*:107, 1952.

severe resistance to insulin. Ketosis ensued and enormous doses of insulin were required to control him—something in the neighborhood of 10,000 to 15,000 units per day. His serum showed insulin inhibitory substances, using the diaphragm technique under about the same conditions to which Dr. Stetten refers. In addition, Dr. Asper conducted parallel studies on the presence of blood precipitins, using polystyrine particles coated with insulin for assay. During the period of refractoriness toward insulin, Dr. Asper demonstrated blood precipitins at high dilutions. These precipitin values roughly paralleled the insulin inhibitors demonstrated by the diaphragm technique. Following treatment with cortisone and ACTH, the ketosis and insulin resistance disappeared and the patient was restored to normality without the need of insulin. Simultaneously there was a complete disappearance of inhibitory bodies, and precipitins in the serum.

DR. ROOT: The patient just mentioned recalls a case whom I saw with DR. COLWELL. This patient entered the hospital for treatment of diabetes, and was found to have hemochromatosis. During the next few months, when he was under observation in the hospital, he developed insulin-resistance, and was finally taking a large dose, in the neighborhood of 2,000 units. As he developed this resistance, the daily insulin dose increasing steadily, the electrophoretic pattern of the blood changed, particularly with respect to the globulins, which increased steadily. He was finally given ACTH and had an extraordinarily rapid decline in his insulin requirement, until he was finally having insulin reactions on rather small doses The presence of precipitins in the blood was demonstrated In a group of our Boston patients, the presence of specific insulin precipitins have been shown. One patient was a Jewish woman of 60 years who had formerly been a mild diabetic, taking only 10-12 units of insulin a day. Over a period of six to eight months she developed insulin-resistance, and had to take 2,000 units of insulin daily.

In various studies we have found that insulin labeled with radioactive iodine is absorbed very slowly from subcutaneous sites in some patients.

On the other hand, when given insulin by vein, a much more

rapid effect on blood sugar levels by normal respiratory quotient was obtained than was observed on a very much larger dose of insulin given subcutaneously. The precipitins of the blood in one patient were studied by Dr. Lowell. He felt that this was perhaps a species effect and that the administration of human insulin might give a better result. However, when this patient was given insulin derived from human pancreas, there was no more marked effect than had been observed with the ordinary commercially prepared insulin. Another patient went into diabetic coma while under treatment on the ward. He had received 400 units of insulin the day previously and yet went into coma within 12 hours. In his case, specific insulin precipitins were present. Some of our patients with insulin-resistance have continued under treatment for a period of several years. Then the resistance has gradually receded. These are patients taking 2,000 to 4,000 units a day. In some cases we thought the insulin-resistance had been initiated or set off by an infection, but in many other cases no infection was demonstrable, nor could we demonstrate the presence of any overactivity in such endocrine glands as thyroid, adrenal or pituitary.

Dr. Gordon: I think the clinicians are confronted almost every day by insulin-resistance, which varies in its intensity from a mild condition, often associated with ketosis, and infections as Dr. Root has indicated, and very rarely associated with the extreme resistance that occurred in the case cited by Dr. Stetten. There are certain well known mechanisms, endocrine in nature, which will affect insulin sensitivity, and these are familiar to everyone. They involve the adrenal cortical hormones, possibly the thyroid hormone, and they certainly involve the anterior pituitary, we assume through growth hormone or its "diabetogenic component." Certain types of juvenile diabetics show extreme liability in their control, so that using the same dose of insulin every day and precisely the same dietary intake, they may fluctuate from insulin shock to severe ketosis. It appeared to us long ago that there must be some metabolic defect in addition to a simple lack of insulin. We set about to determine whether we could identify any intrinsic mechanisms that might be responsible for this. This is a long story. However, it did appear that adrenal cortical hormones,

possibly of the compound E and F type might be in some way responsible, since suppression of their own adrenal cortices by a constant dose of cortisone from the outside, administered as a therapeutic agent, very often but not always levels this off and reduces the instability of these patients, without making their diabetes worse in the sense of increasing insulin requirement.

This is one variety of insulin resistance, which in my opinion is an entirely different sort of phenomenon. I have seen it on a few occasions, and have studied possibly three cases requiring many thousands of units over a very short time. In all of these (all of them died incidentally), at autopsy there was evidence either of hypothalamic or pituitary damage Since we have evolved to the point now where we must consider the hypothalamus and the pituitary as a functioning unit, I would feel that this might be circumstantial evidence supporting Dr. Stetten's feeling that this material might be of anterior pituitary origin.

DR. BEST: I take it that we are all familiar with* Dr. Maloney and Coval's paper on the production of diabetes in mice by injection of guinea-pig anti-insulin serum. The mice showed elevated blood glucose levels, ketonuria and loss of weight.

DR. KINSELL: It has seemed to us that insulin resistance can be many different things, having varied physiological connotations. One might for the present over-simplify, and divide insulin-resistant states which occur in the human, into (1) those which are associated with a tendency to ketosis, and (2) those which are not. Acromegalics and patients with Cushingoid syndromes may have a high degree of insulin resistance, with a surprising lack of tendancy to hyperketonemia and ketonuria. The blood sugar may at all times exceed 300 mgm/100 ml. It would seem to us, in terms of current concepts, that such people, despite their lack of response to administered insulin, must be making insulin. Very possibly they are making tremendous amounts of insulin. On the other side of the fence are those forms of insulin resistance which are characterized by a major tendency to ketoacidosis, or which

* Maloney, P J, and Coval, M · *Biochem J.*, 59:2, 179, 1955

appear during ketoacidosis, the type of patient that Dr. Stetten has described. In addition to abnormal pH, potassium depletion may play a part. Some juvenile diabetics, during the administration of pharmacologic amounts of potassium, sufficient to bring the serum levels to 6 or 7 milliequivalents, show a major decrease in insulin requirement. Dr. McQuarry and his associates some years ago reported that some juvenile diabetics who were receiving relatively large amounts of sodium, had a major decrease in insulin requirement. *The amount of sodium required resulted in* extreme edema. It is our impression that since we have administered potassium *early* in the management of patients admitted in diabetic coma, the rapidity with which insulin resistance disappears is very marked, as compared to the era when we were not "potassium sensitized."

DR. WILLIAMS: I would like to ask Dr. Stetten if he considers that the insulin antagonizing material that he studied in patients with diabetic acidosis was the same type that Dr. Stadie described? Many of the patients who require enormous quantities of insulin do not have acidosis. I think that Dr. Stetten has shown that the interfering material in the diabetic acidotic patient is different from the protein binding phenomenon. I might say that we studied one patient who required about 3000 units of insulin per day, whereas for many months prviously he had required only about 15 or 20 units a day. We found that the capacity of the plasma protein to bind insulin was no greater than in the average diabetic who had had insulin treatment over long periods of time. I might say also that I have had related to me experiences of other physicians with patients who required several thousand units of insulin per day, who with corticosteroid treatment had a decreased insulin requirement. Indeed, even with no insulin or cortical steroid, some of these patients had hypoglycemic reactions; in one case there were hypoglycemic convulsions on several different days, following cessation of all therapy.

DR. STETTEN: There is one other possibility that we have considered but not ruled out. In view of the frequent association of in-

fection with ketosis, the inhibitor we were dealing with, the antagonist, might be of microbial origin. Therefore in one of our patients who had a nice superficial abscess, we have isolated the organism. We could demonstrate no inhibitory activity, in the culture medium. We have looked for patients, and are still looking for patients, with persistant insulin resistance, or what is perhaps more important from our point of view, high tolerance for insulin. We have had one acromegalic, who was apparently almost completely insulin tolerant, who was finally being treated with no insulin at all. He did not develop ketosis but had a persistent hyperglycemia and glucosuria. In the serum of this patient we were unable to demonstrate any antagonists. We had serum from one tubercular patient from Dr. Ralli's service in New York, who was receiving 1500 units of insulin a day. Here also there was no demonstrable humoral antagonist to insulin by the method which we have employed. It has been our experience, and as I gather is the experience of others who have watched such patients, that insulin resistance often evaporates under observation, patients doing well on very moderate doses of insulin after a few days of hospitalization.

DR. BEST: There is the phenomenon, which I think you all know about, which is observed after giving huge doses of insulin, hundreds of units, to normal animals. Frequently no effect on blood sugar is seen. I remember doing this in the summer of 1921, giving huge doses to dogs and finding no fall in blood sugar at all. More recently Dr. Salter with me has been studying this in rats. He has been hunting for the insulin. It is not excreted and he can't find it in the tissues. There may be no fall in blood sugar at all when you give 15 or 20 or 30 times as much as the optimum amount required to produce hypoglycemia

DR. STETTEN. We hear of patients in psychiatric hospitals who are *ad initio* almost completely resistant to administered insulin, though not diabetic. We are trying to secure plasma from such a patient who was given 1500 units of insulin with no fall in blood sugar. This is perhaps similar to the situation which has been

described. As to whether our material is the same as Dr. Stadie's material or not, this I can't say. It was a characteristic reported in the Marsh-Haugaard paper, that the serum, if I recall correctly, had to be treated with the insulin first. If the diaphragms were treated with the serum first, and then washed, and then subsequently treated with insulin, an insulin effect was secured. With our material, if this is an important indication of difference, it makes no difference which of the three agents are used first, second, or last. In other words, we can treat the diaphragm with serum, wash it, then treat it with insulin, and we fail to get an insulin effect. This may or may not constitute a point of difference.

DR. STADIE: Our experiences appear to differ somewhat. Pre-treatment of the diaphragm with serum had no effect upon the subsequent behavior of the diaphragm with respect to insulin. In our experience, the serum to be assayed had to be admixed with insulin in order to demonstrate its inhibitory effect by the diaphragm technique.

DR. HOUSSAY: These experiments about the humoral factors of resistance to insulin are very interesting. However, not all of the resistance to insulin is due to humoral factors. Many years ago we demonstrated that an animal injected with pituitary extract developed insulin-resistance.

In a number of animals we exchanged about 85 or 90 percent of the blood of the animals. They maintained their resistance to insulin, and the controls had no resistance to insulin. The blood was new, but the resistance of the tissues to insulin was maintained.

DR. GORDON: I have just one more comment Dr. Conn, I believe it was, produced a considerable amount of insulin resistance, at least insulin insensitivity, *in vivo* in humans, and I believe in animals as well, by using reduced glutathione or cysteine. I wonder, therefore, if it is sensible to suggest that this possibility should be investigated in relation to the material that you are using.

DR. STETTEN: The reduced sulfhydral compounds are certainly not ruled out by anything we have done. I question whether the material is of low molecular weight.

DR. BEST: Giving BAL sometimes lowers the blood sugar. Was this tested?

DR. STETTEN: No. In general we have not had access to the patients, only to the serum of those patients.

VIII

THE ADRENAL CORTEX AND ENERGY METABOLISM

By E. B. Astwood

There is no need to remind this assemblage of experts that complexities surround the subject we are now about to discuss. Nor is it necessary for me to disclaim extensive experimental experience in this field: it is probably for this reason that I was invited to open this discussion. It has been of great interest to follow the work of many of you, and I hope that it will be possible to touch on those phases of the subject which will invite discussion so that we can hear firsthand of the detailed work that has been carried out. It would be vain to attempt a review.

In this sphere of adrenocortical physiology, as in so many others, it is most difficult to decide which are primary or direct actions and effects, and which are secondary or remote. To study a normal physiologic process it is desirable that all processes in the body be normal except perhaps the one under investigation, but in the instance of adrenal physiology this is virtually impossible. The function of most organs and tissues is conditioned by corticoids and measurements on a single process may yield little information about the part played by the adrenal hormone on that process.

To study the actions of adrenal corticoids on energy metabolism it is often necessary to use adrenalectomized animals and to employ methods which subject these fragile creatures to most unfavorable conditions. Sometimes experiments designed to measure the influence of corticoid deficiency on a metabolic process in reality measure the influence of a failing circulation. Indeed the physiology of adrenal insufficiency may masquerade as the physiology of shock.

Evidence in favor of an action of the adrenal cortex in the regulation of one or another phase of energy metabolism has been

derived from studies on the defect expressed in adrenal insufficiency as contrasted to the state of corticoid excess. One would expect that hormone excess would result in changes which are opposite to those attributable to deficiency but, unfortunately, this is not always the case.

For example, if we had never seen adrenal insufficiency or Addison's disease and were to try to create the picture as the opposite number of Cushing's syndrome, we would invent a monstrous clinical disorder. We would be correct about the changes in blood volume, blood pressure, size of the heart, body weight, appetite, body hair, electrolytes, carbohydrate and insulin tolerance. But our Addisonian lady might be pictured as a very Hercules with great muscles, marble bones, pachydermatous hide, tireless energy, great sexual vigor, and the opposite of amenorrhea, whatever that may be. She would be immune to bruising and trauma, immune to all viral and microbial infections, and immune to gastrointestinal ulceration, perforation, or hemorrhage. We would have made no mention of dermal pigment. We may, of course, have decided that the Addisonian would be so prone to a host of allergic, rheumatic and vascular diseases that the organism would not be able to survive for long enough for the disease to express itself.

This example overstates the case but it does seem to follow that overdosage effects may not be reliable guides to the normal action of a hormone.

Under ideal environmental conditions and with proper diet, extended survival is entirely possible in the absence of the adrenals. Energy can be derived from the common foodstuffs, fat can be formed from carbohydrate or protein, glycogen and depot fat can be used for fuel, and it would probably be difficult to detect any abnormality in carbohydrate, fat, or protein metabolism without imposing some stressful procedure, such as a fast.

In the normal individual, food eaten at each meal is partly burned, partly stored; before the next meal part of the store must be mobilized for use. While glycogen, the most labile energy store, plays a part in permitting periodic feeding, it is insufficient and must be supplemented by a cycle of lipogenesis and adipokinesis. When the fat cycle is operating properly, glycogen stores are prob-

ably conserved and under normal circumstances there is little need for a breakdown of tissue protein to provide for sugar synthesis. Corticoid deficiency expresses itself prominently upon fasting *through a slowed rate of adjustment to the proper handling of* stored energy sources. Mobilization and consumption of fat proceeds at a slower pace at first and only when carbohydrate stores are exhausted does the organism begin to draw heavily upon fat for fuel. From the outset, mobilization of tissue protein is impaired, insufficient amino acids are freed for degradation and sugar formation, and the individual suffers sugar deficiency. Under favorable conditions adjustment is made to a more rapid utilization of fat, permitting survival for extended periods of starvation in the face of a continuing struggle to make enough sugar.

Of the several defects in organic metabolism seen in adrenal insufficiency and corrected by therapy with corticoids, several have their counterparts in the state of corticoid excess. It is instructive, though that in most instances the reversal is incomplete and often distorted. In adrenal insufficiency fat cannot be mobilized rapidly but nonetheless fat stores tend to become slowly depleted, corticoid excess may lead to lipemia and fatty liver but in some species, including man, promotes adiposity. Ketogenesis may be accelerated in both instances and in neither state does there seem to be any great difficulty in the utilization of amino acids. Sensitivity to insulin is reduced to normal by corticoids but great resistance is not ordinarily seen in corticoid excess. Diabetes, if it occurs, is usually mild; this contrasts in degree with the amelioration of diabetes mellitus following adrenalectomy.

The one metabolic process which seems most consistently to be impeded in adrenal deficiency, and accelerated in proportion to the degree of corticoid excess, is proteolysis of cellular stores. It would appear that liberation of amino acids from body protein is a response which comes nearer than any other to being a direct action of adrenocortical steroids. How this action is brought about cannot even be guessed Assuming tissue proteolysis to be a primary consequence of corticoid action and assuming that all organ functions be normal and unaffected by the experimental conditions imposed, can all other findings be reconciled with this single primary role? Actually I think not; we must still presume that

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steroids in the plasma and none produced by the injection of ACTH, if one injects cortical steroids, he finds that these are excreted in the urine as the glucuronides, at least to a considerable extent. Therefore, I think that the observation is probably not a distinctive one from the qualitative standpoint, and if it deals primarily with phenols, it must involve some reaction other than the formation of glucuronides generally.

DR. LEVINE: I was very much intrigued by the way Dr. Astwood treated the subject. He arrived at a possible primary effect of steroids namely that of protein mobilization. I wonder whether this also, is not a secondary effect, in view of Ingle's findings that one does not need extra corticoids to get extra nitrogen excretion, either during fasting, or other stress. Even lymphatic tissues, which do degenerate under the influence of corticoids, can do so in their absence, under the influence of certain stresses.

DR. ASTWOOD: The only demonstrated form of stress, I believe, is the administration of estrogen.

DR. SAMUELS: In connection with that problem, Dr. Levine, I think that the experiments of Dr. Dougherty indicate that in the absence of cortical steroids there is no disintegration of lymphoid tissue in his mice. However, the amounts of compound F that are necessary to produce a graded disintegration are very small. He has established a definite relationship between the dosage of cortisol and the effect of the injection of histamine.

DR. GORDON: I would like to ask if there is any enzymatic documentation or confirmation of the observation that Dr. Astwood reported to us in the intact adrenalectomized animal of an inhibition of the release of amino acids from protein stores? One would think in terms of the activation of cathepsin or something of that sort by these steroid compounds. Have any of these studies ever been done on enzyme systems?

DR. STETTEN: It is my recollection that hepatic arginase has been shown to be increased by the administration of cortisone.

adrenal corticoids play a supportive part in permitting normal rates of response of many reactions. Here, it would seem, corticoids permit the normal functioning of cells, be they liver cells, muscle cells, fat cells, or glandular cells in which these chemical transformations are taking place.

Lysis of tissue protein as a major action of corticoids, though seemingly a direct one when considered at the level of energy metabolism, may still be an indirect action. The primary action may be one which leads to the entire disruption of cellular structure with the release of all cellular constituents. Witness the destruction of lymphatic tissue and the complete dissolution of the thymus following excessive corticoid action; and the increased excretion in the urine of intracellular electrolytes and products of nuclear disintegration.

Considerations based upon observations which have been summarized lead to the conclusion that the site of action of the adrenal cortical secretion cannot be localized at any one known chemical process or enzyme action in the body; the mechanism of action appears to be one influencing the function and survival of the intact cell.

DISCUSSION

DR. STETTEN: I really have nothing further to add. I was intrigued with the hypothetical disease state that Dr. Astwood described, and perhaps, if we keep on looking long enough, one will turn up. There is an observation in the Hungarian literature to the effect that in the adrenalectomized rat given phenol, detoxication proceeded exclusively by sulfation, while in the normal rat, phenol is detoxified partly by sulfation and partly by glucuronide formation. The implication of this observation is that the capacity to form glucuronide, of phenol at least, in the rat is totally dependent upon the presence of the adrenal. I was wondering whether this was true, and if it is true whether it is significant. There are a number of rather tenuous relationships that one can imagine between glucuronide metabolism and the adrenal cortex.

DR. SAMUELS: I think I might have one comment to make. In a completely Addisonian individual, with no measurable adrenal

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atoms derived from such sources as acetate or tagged higher fatty acids unquestionably wind up in carbohydrate. By well known metabolic reactions, they form acetyl-Co A and as such enter the Krebs' cycle. Any member of the Krebs' cycle may be converted into carbohydrate. However, that does not mean net synthesis of carbohydrate from fat. The central thing is this: To get new net formation of carbohydrate from fat, there must be a combination of the two carbon fragments, methyl group to methyl group, thus producing a new four carbon dicarboxylic acid. This is the so-called Thunberg reaction. This four carbon dicarboxylic compound would thus be a new member of the Krebs' cycle stemming from fat. This would mean a *net* new formation of carbohydrate. But the Thunberg reaction has never been demonstrated in mammalian tissue. In other words, no metabolic pathway of fat to carbohydrate via acetyl-Co A formation has been demonstrated. To recapitulate: To incorporate carbon from fatty acid into glucose, a two carbon fragment from the fat condenses with a pre-existing four carbon compound originating from carbohydrate. This new member of the Krebs' cycle may be converted to carbohydrate. On balance, one carbohydrate molecule has been put in and one carbohydrate molecule has come out and there is not a net gain.

There is one other pathway possible which is attributable to Sicoloney, for which confirmation has not been published as far as I am aware. This is a sequence of reactions, postulated largely on the basis of some isotope distribution studies, in which acetone, under some circumstances, is oxidized permanently to pyruvic acid If this actually happens, and as far as I know this is an isolated publication (perhaps Dr. Lardy may recall other publications), it would be possible for two acetyl fragments arising from fatty acid to go to acetoacetic acid and to acetone, three of the four carbon atoms being glucogenic. Whether this actually happens or not I am not certain

DR. LARDY: I think there is pretty good documentation for the pathway, but I don't know whether it has any significance as far as quantity of material is concerned. Fudney has shown that acetone can be a precursor of propanediol, and LePage has shown

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to be well, but in a few hours they develop signs of insufficiency from which they die three to six days after the operation. After the results of Dr. Guspide, we used cortisone and hypocortisone in the post operative care for a week, and from that time until now not one of 29 hypophysectomized dogs has died post operatively.

I would like to emphasize in regard to the adrenal, the remarkable action called permissive or conditioning or supportive. For example, estrogen produces inhibition of the growth of hair, but not if the animal is adrenalectomized. However, if the animal receives only a very small quantity of cortisone, inhibition by estrogen is produced again. This has been studied by Ingle and others. Thyroxine in the adrenalectomized animal, usually does not raise the basal metabolism, but with a very small quantity of cortical extract the action of the thyroid hormone is restored. In the adrenalectomized animal treated with growth hormone it is very difficult to obtain hyperglycemia or diabetes, but with the corticoids this carbohydrate effect again reappears. There are many instances of the so called permissive action of these hormones. After adrenalectomy, as was demonstrated by Elliott a long time ago, and subsequently by many others, the reaction to histamine and to noradrenaline are not normal. By giving a small quantity of corticoids the action again becomes normal. There is also a decrease of resistance to many toxic substances, particularly those which can produce hypotension or increased capillary permeability. The common fundamental mechanism of all these phenomena we don't know yet, but it is probably one of the most important functions of the adrenal. Without it, resistance, especially during emergencies, is strikingly diminished.

Dr. Levine: Dr. Samuels, I would like to point out in regard to insulin sensitivity something which was done first by Leslie Bennett in the hypophysectomized animal, and then we did it in adrenalectomized animals. The insulin sensitivity of both of these preparations is high, higher in the hypophysectomized than in the adrenalectomized animal When insulin sensitivity is tested in the hepatectomized preparation, this sensitivity returned toward normal. At the time I did this work I had a very ready and facile ex-

that propanediol phosphate can, in turn, be converted to lactic acid in tumor preparations, so the pathway is there, but most investigators think that it is only a minor pathway as far as quantity of carbon is concerned.

DR. KINSELL: In the light of more recent studies, in the non-diabetic, using specific mixtures of protein and fat, we have some reason to feel that the inhibition of hyperketonemia brought about by corticoids could be explained to a significant degree by the acceleration of neoglucogenesis from protein. I think we will, at least for the present, accept your concept without serious argument, Dr. Stadie.

DR. HOUSSAY: The idea that in the hyperglycemia of diabetes produced by corticoids, the sugar has come only from protein has been abandoned, because many investigators have demonstrated in a quantitative way that the amount of nitrogen excreted is insufficient to account for the quantity of sugar found in the urine. Dr. Stetten has mentioned the fact that there is an over production of sugar due to increase of gluconeogenesis, six or seven times larger than in normal animals, but we don't know exactly what is the origin of the glucose produced by action of corticoids. The sensitivity to insulin in the adrenalectomized animal is much less than in the hypophysectomized animal. If you have the two operations in one dog, you have the same sensitivity as after hypophysectomy alone. The corticoids produce very striking resistance to insulin. That was shown first by Ingle and then afterwards confirmed by many others. One interesting fact in the hypophysectomized animal is the rapid and striking diminution of the 17-hydroxycorticoids in the adrenal venous blood. I remember that in our laboratory Dr. Guspide found that about 90% reduction in adrenal secretion of 17-hydroxycorticoids occurred within only a few hours after hypophysectomy. Dr. Farrell has found the same results in dogs. Here again is a demonstration that the pituitary action on the adrenal cortex is extremely important. I also remember that my hypophysectomized dogs (I have operated about 900 in 45 years), die in a proportion of about 70% during the first week after the pituitary extirpation. At the beginning they seem

DR. HOUSSAY: Dr. Lardy, there are many functions served by corticosterone in the rat.

DR. GROSS. Since adrenalectomy in the rat does reduce the effectiveness of thyroid hormones, there must be a permissive product being produced in the rat.

DR. LARDY: Cortisone, which is not the preponderant hormone by any means, is capable of supporting the effectiveness of thyroxine.

DR. GROSS: It is a matter of species variation I suppose.

DR. SAMUELS: I would like to say a word in regard to this observation of Dr. Lardy's. One of the factors to be considered in the use of corticosterone in a study of this type is that corticosterone is much more rapidly metabolized than is cortisone. Therefore, the actual production and turnover in the rat is very large, and there is a serious question whether any of these treatments with corticosterone have really maintained the normal levels which are maintained by the rat under normal conditions. Another point that I might bring up is that these substances are metabolized in the peripheral tissues. What the active compound is, I'm afraid we don't know at the present time. This sounds so familiar in this meeting that I feel I am being just repetitive.

DR. GRIFFITH: I would like to support what Dr. Levine had to say about the types of protein in the body. This has to do with the question of the source of amino acids that are involved in gluconeogenesis. I don't see how we can escape accepting the idea proposed by Dr. Madden and Dr. Whipple that there is deposit protein, protein in a form which may not necessarily occur in muscle, certainly not in muscle fibrils and presumably not concerned with muscle function. This is the tissue protein that accounts for the temporary period of negative nitrogen balance if the nitrogen intake is reduced from 20 to 10 grams. This loss of nitrogen is not associated with any weakness of the organism. I

planation, namely, that the insulin sensitivity was due to decreased gluconeogenesis, and therefore removal of the seat of gluconeogenesis eliminates the sensitivity, but I doubt that this hypothesis would explain it all.

DR. SAMUELS: I am impressed with the fact that, from the standpoint of the biochemist, we tend to be too physiological in our use of the term protein. When tissues break down, there is probably a breakdown of active molecules of one sort or another, or in other words enzymes. The effect which is called gluconeogenesis is not necessarily a uniform breakdown of some general protein, all of which has the same activity. Certainly there is a difference in the relative breakdown of proteins in different tissues. We have different types of situations bringing about so-called gluconeogenesis with breakdown of protein. In fasting, one finds a large loss from the liver, and loss from muscle tissue follows. When we have gluconeogenesis due to the action of cortisol, the protein content of the liver will rise while the amount of protein in the muscle tissues is going down. The heart muscle does not change. So we are affecting different proteins in different ways. It would seem that some of the cortical steroids effect certain enzyme systems which are less essential. The "building material" is picked up by other more essential enzyme systems.

DR. LARDY: I suppose that if all of the other hormones are affecting cell permeability, maybe the adrenal cortical hormones could be affecting permeability of muscle cells, with a resultant leakage of protein from the cell. The question I had was with regard to Dr. Houssay's comment on the permissive action of the adrenal cortical hormones for thyroxine effects In the rat it has been demonstrated that the cortical secretion is corticosterone, with only minor quantities of cortisone or hydrocortisone being produced. Dr. Richard Doisy has tested corticosterone as an agent which might support the action of thyroxine in the adrenalectomized rat, and it seems to be completely ineffective. Why is the rat making corticosterone in such large amounts if it isn't an agent which will support the other endocrines in a permissive manner?

DR. GROSS: There is a parallel to this situation in the thyrotoxic rat in which despite the fact that one gets depresssion of growth, there is increase in size of the kidney and also the liver, and apparently, and I think Dr. Houssay would agree, this is probably mediated through the adrenal as well.

DR. KINSELL: The effects on protein tissue, and the relationship to protein catabolism and anabolism cannot be divorced from the effect on electrolytes, with particular reference to potassium. Dr. Leutscher, would you like to carry that further?

DR. LEUTSCHER: I have been fascinated by our tendency, on being pinned down to one specific organ or secretion, to point to some other organ or other secretion as a possible mediator for the effect of the organ initially under discussion. In regard to the adrenal cortex, one of the problems is that the adrenalectomized animal is very much like the eviscerated preparation in that it has a very limited life expectancy. We must have some minute quantity of hormone present at least for the survival of the whole animal, even though this may not be obvious in tissue removed from the body. As Dr. Astwood has described, it is difficult to define specific isolated actions. There are a number of situations in which adrenal cortical hormones have distinctly measurable actions, which can be described by dose response curves, for example, cortisone on liver glycogen, or aldosterone or desoxycorticosterone on electrolyte excretion by the adrenalectomized animal. These are physiological changes involving the whole animal. When we get down to the cell, we seem to approach a permissive action. I wonder if we could have some description of a permissive action. There are enzyme-catalyzed reactions for which various cofactors are necessary, which might be described as simply necessary; however, if those components were titrated down to a certain point, a quantitative relationship would become apparent. When a situation is described in which corticosteroid is necessary and is assigned a permissive action, is it possible that we are dealing with a necessary quantity of the corticosteroid so small that it is inconvenient to measure or titrate? Is there a sharp cut off point, or is the so-called permissive action potentially titratable?

would wonder if the effect here of the corticoids on gluconeogenesis may not be mainly concerned with deposit protein. This may be the protein which we think of in connection with the catabolic phase, protein which is not functional muscle protein in the ordinary sense.

DR. MADDEN: It is certainly true that evidence has been presented for a labile fraction of body protein, one which will be depleted under stress. Once depleted, further stress results in relatively less destruction of protein, so that despite the fact that there remains a tremendous amount of body protein which might be catabolized, for some reason it is relatively resistant. Whether or not this is *structurally different protein, has never been conclusively demon*strated. Studies (Miller) indicate that certain enzymes in organs such as the liver may be almost completely exhausted by abnormal stress, whereas other enzymes will be only partially or not at all depleted. Thus, qualitative as well as quantitative changes in the protein content of an organ may occur under stress. It is interesting to note that although a larger proportion of liver than of muscle protein is labile, the total amount of labile protein contributed by the liver probably is not as great as that contributed by the much larger mass of muscle tissue.

DR. SAMUELS: I would call attention to the fact that at least in the case of the cortical steroids, as has been observed by a number of individuals, the protein of the liver increases under fasting conditions while that of other tissues decreases.

DR. MADDEN: I think Dr. Zeldis mentioned earlier that he and others in our laboratory have some observations in regard to this, not with direct corticoid administration, but in the inflammatory reaction induced by turpentine injury. The liver protein may increase during fasting and the stress of injury to a level appreciably above the normal fed state. This reaction may have similar mechanisms to those during corticoid administration, favoring a protein mobilization from extra-hepatic sources into the hepatic cells.

metabolism at the surface in the process of its release. This brings up, of course, the question that Dr. Astwood raised. It seems to me that all of these things indicate that the corticosteroids act on some type of catalytic system which has basic importance in a number of processes and is rate limiting at certain levels, and may become the actual controlling mechanism or may be so excessive that some other factor becomes rate limiting. This may be at a phase boundary; in spite of Dr. Stadie's tendency to avoid it we still have difference of phase. That's why we see cells. We know for instance that when the lymphocyte disintegrates, something obviously happens to these interphases. You can see it under the microscope. This then must indicate that, whatever the reaction is, it involves some disintegration of phase separation. That's why I wonder if these reactions could be happening at certain areas of this interphase, leading to these different changes, most of which involve a certain amount of retransfer across membranes. I would like to hear what the rest of the group has to say about this.

DR. HOUSSAY: First, the permissive action is something secondary, because the principal thing is that the adrenal allows the animal, or permits the animal, to remain alive, or to be maintained in life with normal responses. That is the principle of permissive action. The others are probably derived from that. We know the description of the decrease in volume of blood, the loss of electrolytes, the vascular and metabolic changes that are important, and we have seen that we can maintain life in the adrenalectomized animal, with electrolytes alone, but without the extra hormones the animal is not completely normal. What the basic deficiency is in the absence of adrenals nobody knows. The idea expressed by Dr. Samuels about permeability has been expressed many times, in the form of hypotheses on changes about the surface phenomena in cells. These were some of the first hypotheses, and they are still acceptable, though they remain without satisfactory demonstration up until now.

DR. KINSELL: As Dr. Astwood pointed out, one of the effects of the cortical hormones, of the cortisone and hydrocortisone type, is to affect fat mobilization, at least in terms of changes in the serum lipids, and a change in the amount of fat in the liver, or rather

DR. ASTWOOD: This is much the line of thought that was going through my head too. The trouble I have with the supportive, permissive, or conditioning action is that in some reactions, excessively increasing the quantities of corticoids not only permits a normal process but exhibits an excessive effect in the same direction. In almost any reaction of the permissive type that I have been able to think of, an excessive effect can be observed in the same direction by giving an excessive dose. Has anyone an example of a truly permissive action?

DR. LEVINE: Isn't there one that has just been mentioned, the calorigenic action of the thyroid hormone? No amount of cortisone given in excess will increase the BMR by itself.

DR. GROSS: I was under the impression that Dr. Astwood quoted a substance associated with corticotropin, that might have some calorigenic action.

DR. LEVINE: Yes, but that is corticotrophin not adrenal corticoids.

DR. ASTWOOD: It depends upon what you measure. If, instead of oxygen consumption, you measure nitrogen excretion there is an excessive effect.

DR. LEVINE: But not with oxygen consumption.

May I, while I think of it, ask Dr. Astwood a question which steers us a little away from protein? How do you conceive of the mobilization of fat? Does it represent an exit of fat from a cell, or is it a disruption of a cell, liberating the fat?

DR. ASTWOOD: My opinion on this matter would be about as useful as my advice to the electronic expert over there on how to record this program. I have always envisioned fat mobilization as a sort of apocrine secretion; little drops of fat get squeezed out of cells.

DR. SAMUELS: I think that fat mobilization is a specific exchange, and it involves energy. The mobilized fat is not exactly of the same composition as fat in the stores, so there is probably some

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lipids in the liver. Another agent, an hormonal entity, that effects lipids is certainly the thyroid. This affects lipids in fairly impressive fashion. A third hormonal entity is the material which Dr. Astwood finds, I believe, is always present even in very highly purified ACTH preparations.

In so far as energy metabolism is concerned, if we could come out with some beginning clarity of concept, in regard to fat transport, then we might be a little bit further along the way. If you take the possible compounds in which fatty acids may travel, you have the chylomicrons, albumin which can adsorb fatty acids and then the globulins. Of the latter there are probably at least two major components, the alpha and beta lipoproteins. Each one of these occurs in fairly constant amounts. Cholesterol can serve as a carrier of one fatty acid; phospholipid can serve as a carrier of two fatty acids, and neutral fat can serve as a carrier for three fatty acids. If fat is the prime fuel of the body, one is dealing with a system in which there are of necessity a number of different "wagons" so to speak, which can perhaps substitute one for the other. I wonder if you would like to comment, Dr. Astwood, on any thoughts you may have about corticoids per se, or about adipokinin in relation to any of this, or in relation to fat utilization?

DR. ASTWOOD: I think that this is a very good scheme, but I must say that I have no information on how fat mobilization takes place, or on how the adipokinetic effect of pituitary extracts is mediated.

DR. LARDY: At the cellular level there are experiments that I think are pertinent. If one separates mitochondria from other particulate and soluble fractions of liver, the microsome fraction added back to the mitochondria stimulate respiration rather vigorously, and we have found that one material that is responsible for this stimulation is free fatty acid It would appear that any physiological regulating agent which altered the fatty acid content of microsomes would in turn have a profound effect on the over-all oxidation in tissues. When fat is being mobilized, there certainly is a possibility for more free fatty acid to be made available to individual liver cells.

DR. LEVINE: Dr. Fritz, in my department, is continuing work which he started in Lundsgaard's laboratory in Copenhagen, in which he showed that a muscle extract added either to liver slices or liver homogenates stimulates the oxygen consumption of these preparations, and increases the oxidation of C_{14} palmitic acid. Part of this action of the extract can be simulated by the addition of carnitine, which is gamma betaine-beta hydroxy butyric acid (22). In some recent experiments he has produced choline deficiency and confirmed Artom's work that in the choline deficient rat liver, fatty acid oxidation is diminished. While choline *in vitro* does not restore the defect, choline, given to the animal three hours before removal of the liver, does. However, addition of carnitine *in vitro* results in an elevation of palmitic acid oxidation. The question comes up, of course, a very tempting one, whether choline is a precursor of carnitine or perhaps a carnitine compound (acetyl carnitine has recently been described which has something to do with activation of fatty acids. Recent experiments suggest that the augmentation of fatty acid oxidation induced by carnitine *in vitro* is mediated by a mechanism different from that induced by choline *in vivo*.

DR. LUETSCHER: One of the things that has recurred over and over again in our discussion has been the question of the necessity of corticosteroid in the calorigenic action of thyroxin. Thinking it over, I do not understand why there are so few evidences of hypometabolism in the patient with adrenal insufficiency. Secondly, if thyroxin is thus deprived of one of its main effects, why should thyroid then sometimes produce such striking and usually deleterious effects in the animal without its adrenal cortex?

DR. KINSELL: *Whether or not the animal gets thyroid, it will lose both sodium and potassium at an alarming rate, and die in adrenal* insufficiency if you don't do something about it rather quickly. If one could keep an animal going I wonder if you would know if you had an effect on oxygen uptake or not.

DR. SAMUELS: You think the electrolyte is so vital?

[22] FRITZ, I *Acta. Phys Skandinav*, V. 34, Fasc. 4, 1955.

DR. KINSELL: In a survival sense, most vital.

DR. GORDON: It is common clinical teaching in most medical schools. I remember myself teaching many times that if a patient were in both adrenal insufficiency and thyroid insufficiency one must be very careful about giving thyroid replacement therapy until the adrenal has been supported. If this depends upon the potentiation, or permission, of the calorigenic action of thyroxin, then this concept is totally wrong, yet I am sure that all of us in clinical medicine have seen disasters of the kind that Dr. Kinsell referred to. In other words, this seems to refute the fact of the potentiation of these two compounds, even though there is the variable of species difference, I suppose.

DR. ASTWOOD: I just wonder, if you give an adrenalectomized animal large doses of thyroid, it is eventually going to lead to his demise, and I wonder whether the animal might not be so adversely effected that he might be in a state of circulatory collapse at the time that the metabolic tests are being done. One would have to be careful that his body temperature were still normal. Perhaps the failure of the BMR to rise might be due to the fact that the animals are pretty sick.

DR. SAMUELS: I think we have had a tendency here to talk about the adrenal cortex as if there weren't influences of a different nature from different types of steroid structure. I think that the question of the importance of the aldosterone type of compound in maintaining the integrity of systems, while you test for something else, must not be overlooked.

The 1956 Metabolic Conference was adjourned at 4:30 P.M., February fifth.

This Book

HORMONAL REGULATION OF ENERGY METABOLISM

Compiled and Edited by

LAURANCE W. KINSELL, M.D.

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